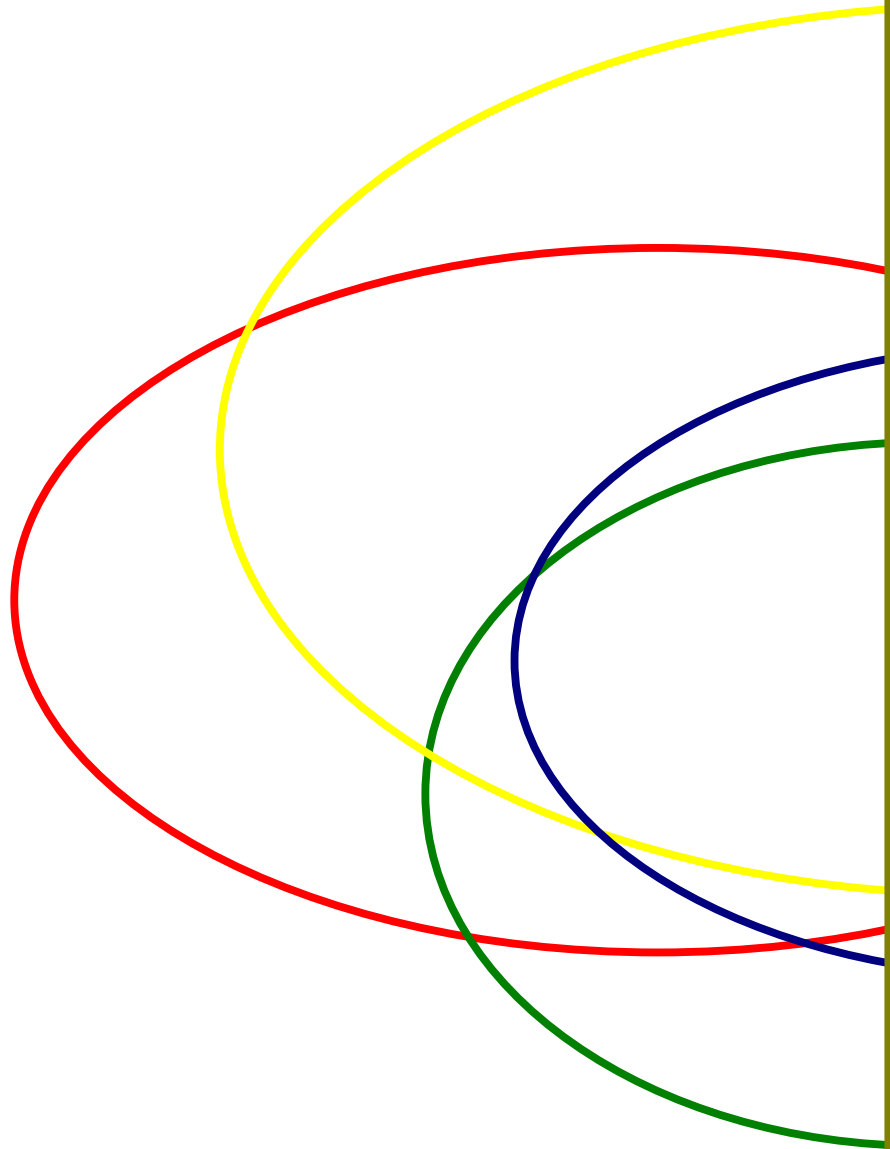


# Caracterización de la Calidad de Sedimentos Afectados por Vertidos de Petróleo

Comparación entre Casos de Vertidos Accidentales (Impacto Agudo) frente a Derrames Continuos (Impacto Crónico)

Carmen Morales Caselles

TESIS DOCTORAL







UNIVERSIDAD DE CÁDIZ  
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**CARACTERIZACIÓN DE LA CALIDAD DE SEDIMENTOS AFECTADOS  
POR VERTIDOS DE PETRÓLEO: COMPARACIÓN ENTRE CASOS DE  
VERTIDOS ACCIDENTALES (IMPACTO AGUDO) FRENTE A DERRAMES  
CONTINUOS (IMPACTO CRÓNICO)**

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HACEN CONSTAR:

Que esta memoria, titulada **“Caracterización de la calidad de sedimentos afectados por vertidos de petróleo: comparación entre casos de vertidos accidentales (impacto agudo) frente a derrames continuos (impacto crónico)”**, presentada por Dña. Carmen Morales Caselles, resume su trabajo de Tesis Doctoral y, considerando que reúne todos los requisitos legales, autorizan su presentación y defensa para optar al grado de Doctor en Ciencias del Mar por la Universidad de Cádiz.

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Memoria presentada para optar al título de  
Doctor en Ciencias del Mar

Carmen Morales Caselles

## *Prólogo*



Fue durante los últimos años de la carrera cuando me di cuenta que lo que me gustaba era la investigación. Entonces me incorporé como alumna colaboradora al departamento de Química Física de la facultad de Ciencias del Mar y Ambientales, donde me acogieron con cariño. A partir de ahí, estuve aprendiendo con y gracias a ellos, los profesores D. Ángel del Valls, D. José Antonio Rubio, D. Abelardo Gómez Parra, D. Eduardo González Mazo, D. Jesús Forja, y los compañeros que estaban con la tesis recién leída o en proceso, Inma, Dori, Rocío, Victor, Mónica, Quique, Idoia, Merche, Loli, Laura, Natalia, Pablo, Carmen, Bibian y Diana.

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Al poco de finalizar la carrera marché a Italia con una beca Argo que me permitió continuar mi formación trabajando en el Centro Ricerche Ambientali di Marina di Ravenna. Allí de nuevo tuve la posibilidad de aprender nuevas cosas referentes a la investigación, gracias a Antonella Iacondini, Federica Abbondanzi y Tiziana Campisi, i capi, maravigliose. Mis compañeros allí fueron estupendos especialmente Agustina, Amaya, Ángeles (Angelita), Alfredo, Marco (Racco), Juan, Luca... También conocí a gente que me hizo la estancia más alegre, los hermanos Frezzati (Annalisa y Pietro), Elena, Cristina, Giorgia, los chicos del “Animal House” (Cecco, Matte, Caprix, Danielle, Gallo, Paco...) y tantos otros amig@s de Ravenna.

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*Lo que sabemos es una gota de agua, lo  
que ignoramos es el océano  
(Isaac Newton)*



*A mi familia*



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# Capítulo 1.

## Introducción, zonas de estudio, objetivos y organización de la tesis

### 1. Introducción

En la actualidad, el petróleo y sus derivados han llegado a ser imprescindibles como fuente de energía y para la fabricación de múltiples productos de la industria química, farmacéutica, alimenticia, etc. Sin embargo, alrededor del 0,2% de la producción mundial de petróleo acaba vertido al mar (Fundación Santiago Rey Fernández-LaTorre. 2003). De manera general se dice que el crudo del petróleo está compuesto principalmente por hidrocarburos, aunque realmente es una mezcla muy compleja de hidrocarburos y de derivados del azufre, nitrógeno, oxígeno y complejos organometálicos, que abarca desde compuestos volátiles de bajo peso molecular, como el metano, hasta compuestos pesados no volátiles, como los asfaltenos. Estos compuestos se distribuyen en familias de hidrocarburos saturados (alcanos y cicloalcanos), aromáticos, cicloalcanos parcialmente aromatizados (naften-aromáticos) y derivados heteroaromáticos (Fundación Santiago Rey Fernández-LaTorre. 2003). El mayor o menor porcentaje de hidrocarburos ligeros o pesados, así como las variaciones importantes en concentración de las diversas estructuras moleculares, hace que existan grandes diferencias entre los crudos producidos a lo largo de la geografía mundial (CSIC, 2003a). La teoría más aceptada sobre la formación del crudo de petróleo afirma que es el producto de la degradación, a

través de grandes presiones y temperaturas, de la materia orgánica procedente de restos de animales y plantas. Una vez extraído el petróleo, generalmente se somete a destilación para separar sus componentes y poder utilizarse (Fundación Santiago Rey Fernández-LaTorre. 2003). Dado que los componentes del petróleo cubren un amplio margen de puntos de ebullición, se pueden separar una serie de fracciones de interés económico (gasolina, queroseno, gas-oil, aceites lubricantes, etc), quedando un residuo de menor valor, que se destina a combustible industrial (fuel-oil) o a asfalto (Fundación Santiago Rey Fernández-LaTorre. 2003).

### *Características de los hidrocarburos aromáticos policíclicos*

Los hidrocarburos, tal y como su nombre indica, están formados por átomos de carbono e hidrógeno. Los hidrocarburos más importantes son los alcanos, alquenos, alquinos y compuestos aromáticos. Los hidrocarburos aromáticos policíclicos (PAHs) están compuestos por átomos de C e H ordenados en forma de dos o más anillos de benceno. Hay miles de PAHs, cada uno con un número y una disposición particular de los anillos aromáticos y de los sustituyentes (Sims y Overcash, 1983). Las propiedades físico químicas de los PAHs varían generalmente con el peso molecular. Al aumentar el peso molecular, la solubilidad en agua disminuye, y el punto de fusión, el punto de ebullición y el log Kow (coeficiente de partición octanol-agua) aumentan suponiendo un aumento de la solubilidad en las grasas, una disminución en la resistencia a la oxidación-reducción, y una disminución de la presión de vapor (tabla 2.1). Por ello, los PAHs varían su comportamiento en el medio ambiente en función de su peso molecular (Eisler, 2000). Así, por ejemplo, el Benzo[a]pireno debido a su baja solubilidad y presión de vapor y elevado Kow se va a incorporar principalmente al suelo y a los sedimentos, quedando menos del 1% en el resto de los compartimentos ambientales (agua, biota, aire y sólidos

en suspensión). Otros PAHs, como los de 2 y 3 anillos (Naftaleno, Acenafteno, Antraceno, Fluoreno, Acenaftileno y Fenantreno), suelen en cambio encontrarse predominantemente en el aire (Baek et al., 1991). Así, se ha podido comprobar como la baja solubilidad de los PAHs y su alta afinidad por el carbono orgánico hace que en el medio acuático más de 2/3 se encuentren asociados a partículas, eliminándose los de medio y alto peso molecular por adsorción al sedimento principalmente y los de bajo peso por volatilización y biodegradación.

**Tabla 2.1.** Algunas de las propiedades químicas del Naftaleno, Antraceno, Benzo[a]antraceno, Benzo[a]pireno, Benzo[g,h,i]perileno.

<i>Compuesto</i>	<i>Nº anillos</i>	<i>Peso molecular</i>	<i>Punto de fusión (°C)</i>	<i>Solubilidad en agua (mg L<sup>-1</sup>)</i>	<i>Log K<sub>ow</sub></i>
Naftaleno	2	128	80	30.0	3.37
Antraceno	3	178	216	0.07	4.45
Benzo[a]antraceno	4	228	158	0.014	5.61
Benzo[a]pireno	5	252	179	0.0038	6.04
Benzo[g,h,i]perileno	6	276	222	0.00026	7.23

El origen de estos hidrocarburos aromáticos policíclicos en el medio ambiente es muy diverso, aunque básicamente se pueden identificar tres fuentes distintas (Eisler, 2000):

- Origen *pirolítico*, procedente de la combustión incompleta de la materia orgánica, reciente o fósil, bien por causas naturales (incendios de bosques, erupciones volcánicas, etc.) o antropogénicas (utilización de combustibles fósiles, incineración de residuos, emisiones de vehículos, procesos industriales de gasificación y licuefacción del carbón, etc.).

- Origen *petrogénico*, producido por vertidos accidentales o intencionados de derivados del petróleo. Se caracterizan por ser mezclas complejas formadas por compuestos con cadenas alquílicas de hasta 5 o 6 átomos de carbono.

- Origen *diagenético* de la materia orgánica sedimentaria, la cual puede sufrir una serie de procesos geoquímicos naturales, como son la descarboxilación, aromatización, desfuncionalización, etc. para convertirse en PAHs de origen natural. Entre éstos encontraríamos ciertos derivados del criseno y del piceno, así como el reteno y el perileno, aunque estos dos últimos también pueden tener un origen pirolítico.

A pesar de que los PAHs se encuentran de forma natural en el medio ambiente, la mayor parte de estos compuestos presentes en la naturaleza tienen origen antropogénico, llegando a descargarse en el medio acuático alrededor de 230000 toneladas de PAHs al año (tabla 2.2).

**Tabla 1.1.** Principales fuentes de PAHs en el medio acuático.

<i>PAHs totales</i>	<i>Toneladas/año</i>
Vertidos de petróleo	170000
Deposición atmosférica	50000
Aguas residuales	4400
Escorrentía	2940
Biosíntesis	2700

***Comportamiento de los hidrocarburos en el medio marino.***

Tal y como se ha visto anteriormente, el crudo de petróleo está compuesto principalmente por hidrocarburos que varían entre muy volátiles,

que son sustancias ligeras como el propano y el benceno, y compuestos pesados como los asfaltenos y resinas. Una vez que los hidrocarburos se vierten al mar, éstos tienden a volatilizarse o esparcirse en el medio, en función de su composición. Este comportamiento de los hidrocarburos en el mar, va a determinar que sus componentes se distribuyan en los distintos compartimentos ambientales. La forma en que la capa de hidrocarburos se rompe y se disipa en el mar, va a depender en gran parte de la persistencia del fuel vertido. Los productos ligeros como el queroseno, tienden a evaporarse y se disipan rápidamente. Estos tipos de fuel se denominan “no persistentes”. Por otra parte, las sustancias “persistentes”, como la mayoría de los crudos de petróleo, se disipan más lentamente. La International Tanker Owners Pollution Federation, ITOPF (<http://www.itopf.com/index.html>) ha realizado una clasificación de los procesos que pueden sufrir los hidrocarburos una vez vertidos en el medio marino:

**A) Esparcimiento.** Tan pronto como el fuel es vertido en el mar, éste comienza a esparcirse por la superficie del agua. La velocidad a la que tiene lugar este proceso, depende en gran parte de la viscosidad del fuel. Aquellos que sean fluidos, con baja viscosidad se van a esparcir más rápidamente que los que presentan viscosidad alta. La capa de fuel va a romperse por la acción del viento, las olas, y las turbulencias, de forma que las llamadas “manchas de petróleo” se van a disponer paralelamente a la dirección del viento. La velocidad a la que se van a esparcir las manchas de petróleo, también va a depender de otros factores como la temperatura, las corrientes, la marea y la velocidad del viento. Cuanto más severas sean las condiciones meteorológicas, más rápidamente se va a producir el esparcimiento y ruptura de las manchas de petróleo.

**B) Evaporación.** Aquellos compuestos más ligeros se van a evaporar a la atmósfera. La cantidad de hidrocarburo que se evapore y la velocidad con que lo haga, va a depender de lo volátiles que sean los compuestos que lo componen. Así, por ejemplo, si se trata de un fuel pesado sólo se evaporará una pequeña parte. De manera general, se puede decir que aquellos componentes del fuel cuyo punto de ebullición sea menor que 200°C van a tender a evaporarse en las primeras 24 horas. El fenómeno de evaporación se va a incrementar al aumentar el área superficial de la capa de fuel. Las altas temperaturas, la alta velocidad del viento, etc., van a aumentar la tasa de evaporación y la proporción de fuel que sufre este proceso.

**C) Dispersión.** Las olas y la turbidez en la superficie del mar pueden provocar que la mancha de fuel se fragmente en trozos de distinto tamaño. Esto hace que muchos de estos trozos de fuel lleguen a los niveles más altos de la columna de agua. Algunos de los trozos más pequeños se mantendrán suspendidos en la columna de agua, mientras que aquellos más grandes van a tender a volver a la superficie, donde pueden unirse con otros trozos y volver a formar una fina capa superficial. El fuel que queda en la columna de agua tiene una elevada área superficial tras el proceso de dispersión, lo que induce a que se den otros procesos naturales como la disolución, biodegradación y sedimentación. La velocidad a la que un fuel se dispersa, va a depender de la naturaleza del mismo y del estado de la mar, incrementándose si el fuel es ligero, tiene baja viscosidad, y si la superficie del mar se encuentra alterada. La adición de dispersantes químicos puede acelerar el proceso natural de dispersión.

**D) Emulsificación.** Una emulsión se forma cuando se combinan dos líquidos, de forma que uno de los dos queda suspendido en el otro. Se habla de emulsificación del fuel cuando las gotas de agua se suspenden con él. Esto ocurre por mezcla física de ambos componentes y viene determinada por la

turbulencia en la superficie del agua. La emulsión formada suele ser viscosa y más persistente que el fuel original, y hace que el volumen del contaminante aumente entre tres o cuatro veces. Esto ralentiza y retrasa otros procesos que permitirían la disipación del fuel. Aquellos tipos de fuel con un contenido en asfaltenos mayor del 0.5% tienden a formar emulsiones estables que pueden persistir varios meses después del vertido. Si una vez en la costa, las emulsiones aumentan su temperatura debido a la incidencia de la luz solar, éstas van a poder romperse separando de nuevo el fuel y el agua.

**E) Disolución.** Los compuestos solubles del fuel van a poder disolverse en el agua. Esto depende de la composición y del estado del fuel, y ocurre más rápidamente cuando el fuel se encuentra disperso en la columna de agua. Los componentes más solubles en el agua de mar son los hidrocarburos aromáticos ligeros como el benceno y el tolueno. Sin embargo, estos compuestos son aquellos que se van a perder en primer lugar por evaporación, un proceso que es 10 – 100 veces más rápido que la disolución.

**F) Oxidación.** El fuel va a reaccionar químicamente con el oxígeno, bien rompiéndose en fracciones solubles, o bien formando compuestos persistentes que reciben el nombre de “alquitrán”. Este proceso está favorecido por la luz solar (fotooxidación) y va a depender del tipo de fuel y de la forma que se encuentra expuesto a la luz. Sin embargo, es un proceso muy lento. La formación del alquitrán se da por la oxidación de capas gruesas de fuel muy viscoso o por la oxidación de emulsiones. Este proceso forma una protección externa para los componentes pesados lo que hace aumentar la persistencia del fuel. Las conocidas “galletas de alquitrán” que se encuentran normalmente en la costa, son productos de este proceso de oxidación.

**G) Sedimentación.** Algunos componentes pesados tienen densidades mayores que el agua dulce por lo que sedimentan. Sin embargo, el agua de mar

tiene una densidad mayor que el agua dulce y muy pocos crudos de petróleo son lo suficientemente densos como para hundirse en el medio marino. La sedimentación ocurre normalmente por la adhesión del fuel con partículas de sedimento o materia orgánica. Generalmente, las aguas someras están cargadas de sólidos en suspensión favoreciendo el proceso de sedimentación. Cuando el fuel llega a costas arenosas suele mezclarse con arena y otros sedimentos. Si posteriormente, esta mezcla vuelve al mar, probablemente se hunda. Además, si el fuel se quema tras ser vertido, los residuos pueden volverse lo suficientemente densos y llegar a sedimentar.

**H) Biodegradación.** El agua de mar posee una serie de microorganismos que pueden degradar parcialmente o completamente el fuel en compuestos solubles en agua, hasta CO<sub>2</sub> y agua. Cada tipo de microorganismos es capaz de degradar un grupo de compuestos particulares. Sin embargo, algunos compuestos de hidrocarburos son resistentes a la degradación. La eficiencia de la biodegradación va a depender de varios factores: los niveles de nutrientes (nitrógeno y fósforo) en el agua, la temperatura y la presencia de oxígeno. Como la biodegradación precisa de oxígeno para poder realizarse, este proceso sólo va a poder llevarse a cabo en la interfase fuel-agua, ya que el fuel no posee oxígeno. Por tanto, la biodegradación será mayor al aumentar la relación superficie/volumen del fuel (por ejemplo, tras procesos de dispersión).

Los procesos de esparcimiento, evaporación, dispersión, emulsificación y disolución, son los más importantes durante las primeras fases del vertido, mientras que la oxidación, la sedimentación y la biodegradación cobran más importancia después de pasado un tiempo y determinan el destino final del fuel.

### ***Efectos de los hidrocarburos en el medio marino***

Los efectos de los vertidos de hidrocarburos en el medio ambiente marino pueden estar causados por la naturaleza física del fuel o bien por la composición química de éste. Desde el punto de vista físico, la impregnación de los organismos que entran en contacto directo con el fuel va a suponer una de las causas letales más comunes, así como el agotamiento y la ingesta de hidrocarburos. La mortalidad se produce al impedir la respiración, fotosíntesis, o al modificar la resistencia térmica (por ejemplo las aves). Desde el punto de vista químico, cabe destacar los efectos tóxicos agudos y crónicos. Además, las alteraciones a nivel de organismo, y sus consecuencias demográficas, pueden desembocar en cambios en la estructura de las comunidades ecológicas y, por tanto, en una alteración de la red de las interacciones existentes (Fundación Santiago Rey Fernández-LaTorre, 2003).

Los componentes más tóxicos del petróleo son aquellos hidrocarburos de bajo peso molecular que se van a perder rápidamente por evaporación, de forma que no suelen llegar a concentrarse lo suficiente para provocar efectos letales en los organismos. Por otra parte, una exposición prolongada al fuel o a sus componentes puede producir efectos subletales que pueden afectar a las funciones vitales de los organismos como la reproducción, crecimiento o alimentación (ITOPF, <http://www.itopf.com/index.html>).

La toxicidad de los hidrocarburos de petróleo reside básicamente en el potencial cancerígeno de los hidrocarburos aromáticos policíclicos (PAHs). Entre ellos destacan los 16 compuestos incluidos en la lista de contaminantes prioritarios de la Environmental Protection Agency de los Estados Unidos: Naftaleno, Acenaftileno, Acenafteno, Fluoreno, Fenantreno, Antraceno, Fluoranteno, Pireno, Criseno, Benzo[a]antraceno, Benzo[k]fluoranteno,

Benzo[a]pireno, Dibenzo[ah]antraceno, Indeno[123-cd]pireno, Benzo[ghi]perileno (USEPA, 2000).

Una de las características de los PAHs que los hace más peligrosos es su acumulación en los organismos acuáticos desde el agua, el sedimento o el alimento. Así se han encontrado valores de factor de bioacumulación (BCF) entre 10 y 10000 para peces y crustáceos, correspondiendo los valores más elevados a los PAHs de mayor peso molecular (Eisler et al., 2000). Estas sustancias sin embargo, pueden ser rápidamente metabolizadas por los organismos (de 2 a 9 días en peces), lo cual evita la biomagnificación pero puede presentar un problema aún mayor al generarse metabolitos carcinógenos y mutagénicos.

En teoría, todos los organismos poseen enzimas de biotransformación o detoxificación que convierten las sustancias xenobióticas lipofílicas en metabolitos solubles en agua que pueden ser excretados (Neff, 1979). En la Fase I de los procesos metabólicos, los PAHs son transformados en varios productos como epóxidos, fenoles, quinones, dihidrodioles, epóxidos, tetrahidrotroles y tetrahidrotetroles. Los metabolitos intermedios, han sido identificados como agentes mutagénicos, carcinógenos y teratógenos (Sims and Overcash, 1983). La activación de estos mecanismos tóxicos ocurre por hidroxilación o por producción de epóxidos inestables de PAHs que dañan el ADN, iniciando los procesos cancerígenos (Jackim y Lake, 1978; Schnitz y O'Connor, 1992).

### ***Importancia de los sedimentos en estudios de contaminación***

Cualquier sustancia que se encuentre en un medio en concentraciones superiores a los niveles naturales, puede alterar el equilibrio del ecosistema, llegando incluso a destruir el biotopo, a limitar la explotación de los recursos biológicos y a poner en peligro la salud humana. La presencia de una sustancia

en el medio en concentraciones mayores a las naturales se denomina contaminación, y el fenómeno por el que este enriquecimiento de contaminantes produce efectos biológicos, se define como polución.

Muchos de los contaminantes orgánicos e inorgánicos que se originan con la actividad humana, son transportados, tanto en disolución como adsorbidos sobre el material particulado en suspensión, desde las zonas continentales hacia los océanos. Las zonas costeras, y especialmente los sistemas más someros, son los receptores principales de las sustancias contaminantes vertidas a los océanos. Dado el carácter activo de estos compuestos, durante el transporte se incorporan al sedimento a través de diferentes procesos de adsorción o reacciones químicas (Salomons et al., 1987).

Además, se ha demostrado que los contaminantes orgánicos menos polares y los elementos traza más tóxicos reaccionan fuertemente con el material particulado y se acumulan en el sedimento en órdenes de magnitud más altos que en solución (DeValls et al., 2002). Los sedimentos actúan, por tanto, como “trampa” de contaminantes, de forma que éstos reflejan de una forma muy eficaz el grado de contaminación de un área determinada. Este hecho ha llevado a numerosos autores a considerar, desde un punto de vista ecológico, a los sedimentos como un elemento de una importancia trascendental del hábitat acuático (Chapman, 1989; Luoma, 1983; 1989; Luoma y Ho, 1992).

Los estudios de contaminación en sedimentos se basan generalmente en la comparación de los niveles de contaminantes medidos en una zona determinada con aquellos niveles de contaminantes medidos en una zona de referencia que se considera no contaminada. En muchos casos los estudios de este tipo permiten identificar el origen de la contaminación (Luoma y Philips, 1988; Philips et al., 1992; French, 1993) e incluso estimar su biodisponibilidad

(Harvey y Luoma, 1985; Luoma y Bryan, 1978; Arjonilla et al., 1994). Esta metodología, sin embargo, no permite obtener información sobre el fenómeno de polución.

Una gran variedad de organismos quedan expuestos a los contaminantes presentes en los sedimentos. Los animales que ingieren sedimento o detritus particulado como alimento, se encuentran directamente afectados, de forma que estos contaminantes entran a formar parte de la cadena trófica, por lo que otros organismos pueden verse también afectados y se pueden dar fenómenos de biomagnificación del contaminante en los sucesivos niveles tróficos.

La contaminación de los sedimentos no sólo afecta a los organismos de la zona contaminada sino que además puede extenderse a ecosistemas alejados de las fuentes mediante la resuspensión y transporte de partículas (Luoma, 1990).

La definición de toxicidad del sedimento, en su sentido más amplio, está referida a los cambios ecológicos y biológicos causados por los contaminantes que se encuentran incorporados en los sedimentos. En términos toxicológicos, queda definida como la respuesta adversa que se observa en uno o en varios organismos sometidos a una prueba donde se les expone a sedimentos contaminados. Esta respuesta adversa se evalúa de manera objetiva cuando los animales se exponen a sedimentos limpios a los que les han sido añadidas concentraciones conocidas de contaminante ("Spiked Sediment"), o bien cuando son expuestos a sedimentos contaminados recogidos en el medio. (Chapman y Morgan, 1983; Oakden et al., 1984; Kemp y Swartz, 1988; Swartz et al., 1989; Swartz et al., 1990; Meador, 1990; McGee et al., 1993; DelValls, 1994).

La dificultad del estudio de la toxicidad de los sedimentos marinos se basa en que los contaminantes presentes en ellos forman una mezcla compleja

de sustancias que pueden dar lugar a efectos biológicos sinérgicos o antagónicos. Quizás por ello, en la actualidad, aún existe cierto desconocimiento sobre la toxicidad de muchas sustancias vertidas al medio. De hecho, se considera que sólo del 5 al 10% de ellas, han sido probadas en laboratorio para evaluar su toxicidad. De ese número, menos del 1% lo han sido utilizando organismos marinos (Martell et al., 1988).

Algunos gobiernos han diseñado programas para evaluar la calidad ambiental de los sedimentos, y han realizado unas guías numéricas de calidad de los sedimentos (Sediment Quality Guidelines -SQGs-) para cada contaminante (Long and Buchman, 1989; NOAA, 1999; Environment Canada, 2003) basándose en estudios previos de toxicidad. Estas guías se definen como las concentraciones de contaminantes presentes en sedimentos que están asociados o no a efectos biológicos en laboratorio o campo (DeValls y Chapman, 1998). Las guías permiten comparar el grado de polución entre distintas zonas, de forma que puede resultar una herramienta útil en estudios de calidad ambiental.

## **2. Zonas de estudio**

Para llevar a cabo el presente trabajo se escogieron dos zonas del litoral español afectadas por vertidos de petróleo. Asimismo se seleccionó un área de referencia para el estudio.

### ***La costa de Galicia: el vertido del Prestige***

A finales del 2002 el accidente del petrolero monocasco *Prestige* provocó un vertido de 63000 toneladas de fuel oil pesado que se esparció en manchas, más o menos compactas y que supuso una de las 'mareas negras' más dañinas

de las ocurridas en Galicia en los últimos años junto a las causadas por el *Urquiola* (1976) y el *Aegean Sea* (1992). Además, se trata de la tercera marea negra de fuel pesado en aguas europeas en menos de 4 años, después de las provocadas por los petroleros *Erika* (1999) y *Baltic Carrier* (2001). La costa gallega fue la zona más afectada por el vertido y los efectos de la catástrofe se dejaron sentir en el medio ambiente y en la economía y supuso un gran impacto ecológico y social.

La costa de Galicia ha vivido muchos naufragios en sus aguas y es considerada una zona de alto riesgo para los navíos (Bulot, 2003). A lo largo de la historia, Galicia ha sufrido en varias ocasiones la llegada de hidrocarburos a sus costas. Una de las primeras alertas de contaminación por hidrocarburos se produjo el 27 de febrero de 1961, cuando el petrolero canadiense *Andros Fortune*, colisionó con un carguero. Sin embargo, en esta ocasión, el vertido fue muy reducido, ya que sólo se vertió al mar el combustible previsto para el funcionamiento de las máquinas (Bulot, 2003). El 6 de mayo de 1970, el petrolero noruego *Polycommander*, tocó fondo al salir del puerto de Vigo vertiendo unas 15000 toneladas de crudo ligero en la bahía de Vigo. Seis años después, el petrolero español *Urquiola*, colisionó con una aguja sumergida en el acceso al puerto de A Coruña. Se vertieron 110000 toneladas de crudo (Bulot, 2003). El 31 de diciembre de 1978, el petrolero griego *Andros patria*, sufrió una importante brecha liberando unas 60000 toneladas de petróleo que mancharon unos 50 km de la costa de Lugo (Bulot, 2003). Fue casi catorce años después, el 3 de diciembre de 1992, cuando el *Aegean Sea* provocó una nueva marea negra en las costas de Galicia, vertiendo 66800 toneladas de crudo ligero junto a la Torre de Hércules en A Coruña. Este episodio ha servido de base para realizar estudios posteriores en el caso del *Prestige* (CSIC, 2003a).

Sin embargo, el comportamiento del fuel en el medio marino depende, en gran parte, de su composición. Sabiendo que la carga del *Prestige* consiste en

un fuel pesado, cabe analizar los accidentes de petroleros que han originado este tipo de vertido, de forma que nos podamos acercar a los posibles efectos ocasionados sobre el medio marino. Así, por ejemplo, la experiencia adquirida con el vertido de 20000 toneladas de fuel pesado del *Erika* (12 de diciembre de 1999) frente a las costas francesas, ha permitido avanzar en las actuaciones y los estudios del *Prestige* (Hoefer, T., 2003; Le-Cedre, <http://www.le-cedre.fr/>; Ifremer, <http://www.ifremer.fr>).

Los ejemplos de vertidos accidentales de un producto petrolífero pesado en el mar, de un petróleo crudo o de un producto refinado cuya densidad varía entre 0.95 y 1.00 g m<sup>-3</sup>, son numerosos (Le-Cedre, <http://www.le-cedre.fr>). En la tabla 2.3 se resumen algunos de estos accidentes. Este tipo de vertido (fuel pesado) produce extensas manchas, galletas y bolas de hidrocarburos, originadas por la fragmentación del producto durante su deriva marina. Casi siempre, el impacto sobre las aves y los mamíferos marinos impregnados de petróleo es muy elevado. El vertido de este fuel pesado, al presentar unos componentes evaporables y biodegradables minoritarios (menos del 10 % de su masa), suele exigir unos amplios trabajos de limpieza, que se vuelven difíciles debido a la extremada viscosidad del producto. En compensación, éste difunde en el agua escasos componentes tóxicos y, una vez que la limpieza se ha llevado a cabo, los efectos a largo plazo son mínimos (Le-Cedre, <http://www.le-cedre.fr>).

Por otra parte, los fenómenos de sedimentación del fuel pesado van a suponer una entrada de contaminantes a los sedimentos, que más adelante pueden volver a la columna de agua. Este fenómeno se ha comprobado en estudios previos, donde se detectaron nuevos picos de contaminación meses después del vertido, en el caso del *Erika* (Burgeot, 2001) debido a efectos

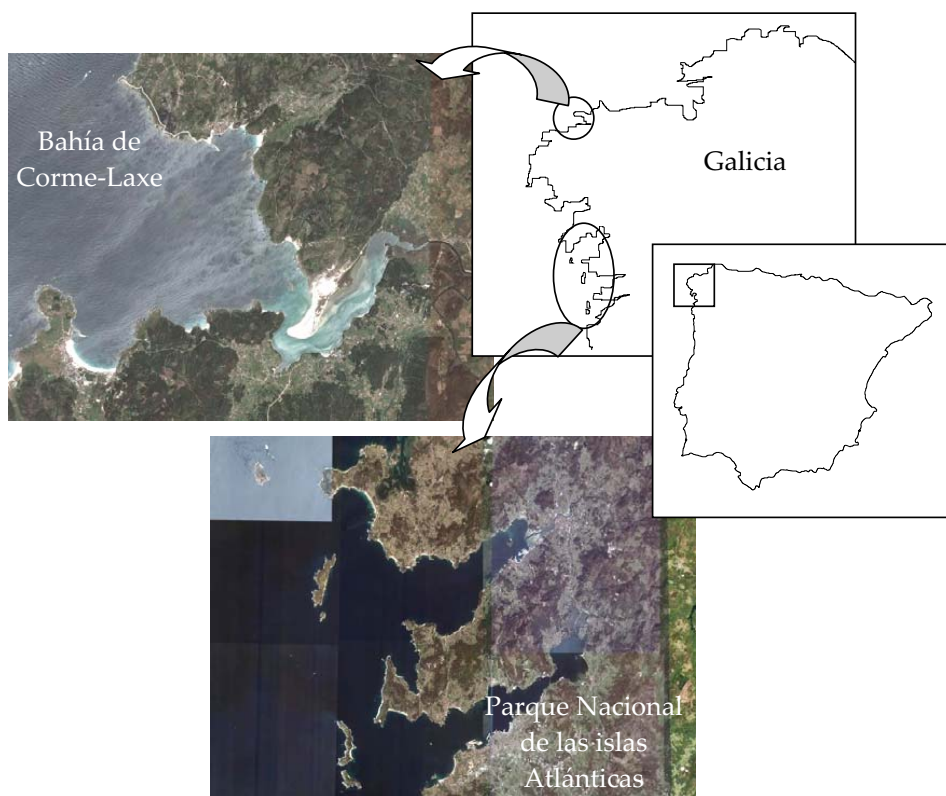
mareales, y a la remoción de fondos por la extracción de arenas, en el caso de *Aegean Sea* (MMA, 1993).

**Tabla 1.2.** Accidentes de petroleros que han vertido al mar fuel pesado en los últimos años (Le-Cedre, <http://www.le-cedre.fr>).

Año	Petrolero	Lugar	Cantidad vertida (toneladas)
2001	<i>Baltic Carrier</i>	Dinamarca	2700
1999	<i>Erika</i>	Francia	20000
1997	<i>Nakhodka</i>	Japón	6.20
1997	<i>Katja</i>	Francia	0.19
1988	<i>Nestucca</i>	EEUU	11
1984	<i>Mobiloil</i>	EEUU	0.64
1980	<i>Tanio</i>	Francia	6500
1976	<i>Bohlen</i>	Francia	6500
1976	<i>Argo Merchant</i>	EEUU	5.70
1972	<i>Tamano</i>	EEUU	0.40
1970	<i>Arrow</i>	Canadá	12
1969	<i>Hamilton Trader</i>	Gran Bretaña	0.64

Los análisis químicos, revelaron una composición del fuel del *Prestige* cercana a la del *Erika*. Los niveles de concentraciones en PAHs en la muestra de fuel del *Prestige* son próximos a aquellos del fuel del *Erika* recogidas en las playas. Sin embargo, parece ser que la muestra de referencia *Erika* de la refinería de Dunkerque es más rica en PAHs. La equivalencia tóxica en Benzo(a)pyrene parece indicar potenciales tóxicos similares entre los dos fuels (Ifremer, <http://www.ifremer.fr/envlit/prestige/indexsp.htm>).

Transcurridos tres años del naufragio del *Erika*, los análisis químicos en los mariscos, mostraron que aún se encuentran huellas del *Erika* en ciertos lugares. Esto muestra la persistencia de ciertos componentes de ese fuel en el agua y en los sedimentos (Ifremer <http://www.ifremer.fr/>) lo que pone de manifiesto la importancia del estudio de la polución a lo largo del tiempo.



**Figura 1.1.** Áreas de estudio en las costas gallegas afectadas por el vertido del petrolero *Prestige* (imágenes tomadas de Google™ Earth).

Tras el accidente del petrolero *Prestige*, los archipiélagos de Cíes, Ons y Salvora, declarados en julio del 2002 (Ley 15/2002) Parque Nacional de las Islas Atlánticas, fueron la barrera natural que frenó la entrada del fuel en las Rías Bajas Gallegas. El director de Parques Naturales reconoció que en una primera

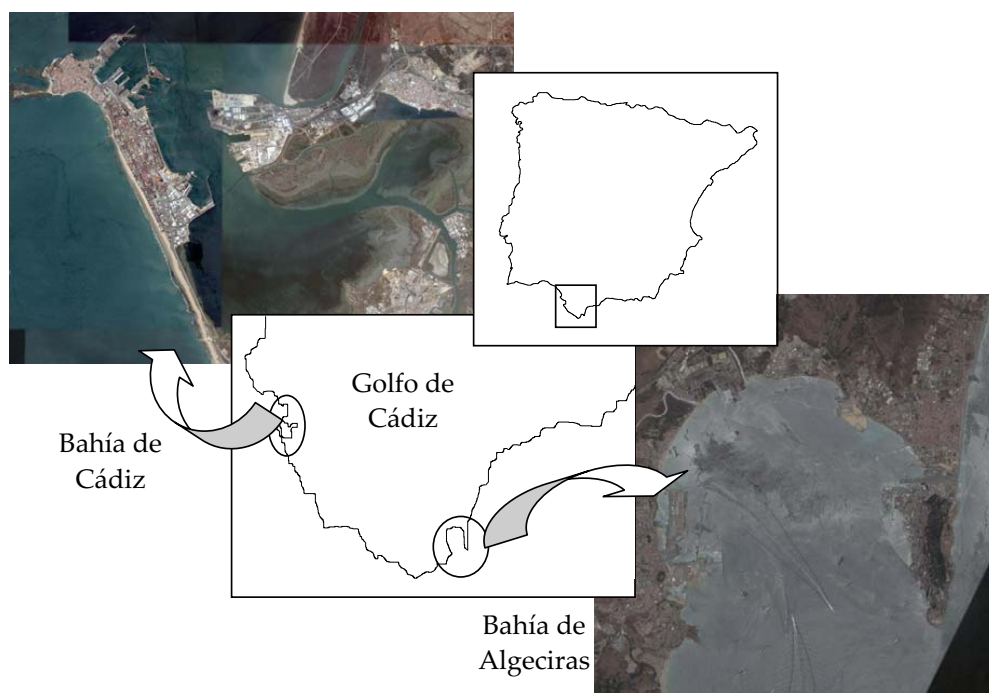
oleada de marea negra, el 85% del Parque Nacional de las Islas Atlánticas resultó afectado, elevándose este porcentaje a más del 90% tras oleadas posteriores de fuel recibidas por el Parque.

El vertido del petrolero *Prestige* también afectó de manera importante a la Bahía de Corme-Laxe la cual era considerada una zona con baja influencia antropogénica y una moderada actividad industrial, destacando sobretodo las tareas agropecuarias y pesqueras de la zona. El polígono de viveros de Corme tiene bateas experimentales dedicadas a la producción de mejillón, vieira, zamburiña y ostra plana. Corme Laxe presenta una influencia oceánica grande y el mayor riesgo está en el tráfico marítimo exterior que transporta mercancías peligrosas y está expuesto a sufrir un accidente marítimo (Diario oficial de Galicia, 2004). Los tipos de hidrocarburos susceptibles de ser vertidos en la zona son principalmente el gasoil de consumo de los barcos, gasolinas, fuel-oil y gas-oil de los transportes terrestres y estaciones de suministro de combustible, residuos oleosos procedentes de reparaciones en puerto y talleres de automoción. Por otra parte, el río Anllóns, que desemboca en la ría, ha sufrido episodios de contaminación por vertidos de purinas procedentes de granjas.

### *El Golfo de Cádiz: La Bahía de Algeciras y la Bahía de Cádiz*

A partir de los años 60, la franja costera de la Bahía de Algeciras se ha visto paulatinamente sometida a drásticas transformaciones provocadas por el creciente desarrollo socioeconómico de la zona. Así, en la actualidad, en sus márgenes se asienta un importante polo industrial integrado por plantas petroquímicas, centrales térmicas, industrias siderometalúrgicas, papeleras, astilleros, además de una intensa actividad portuaria, una creciente presión demográfica y el elevado nivel de transformación de la costa por distintas construcciones (Conradi et al., 1995).

Existen pequeños cauces fluviales que desembocan al interior de la bahía de Algeciras, siendo los ríos Palmones y Gaudarranque los más importantes respecto al caudal (Conradi et al., 1995). La hidrología general de la bahía se ve afectada por la circulación general de las masas de agua atlánticas y mediterráneas a través del Estrecho de Gibraltar, aunque las corrientes que afectan la bahía están causadas principalmente por cambios de dirección en la corriente de marea, vientos, presión atmosférica y la corriente mediterránea superficial (Conradi et al., 1995). Esta bahía presenta una alta tasa de renovación de sus aguas, debido a su proximidad al Estrecho, y a las fuertes corrientes de aguas, lo que da lugar a que los efectos de los vertidos contaminantes se vean notablemente reducidos, al dispersarse en una gran masa de agua (Consejería de Medio Ambiente, 2007).



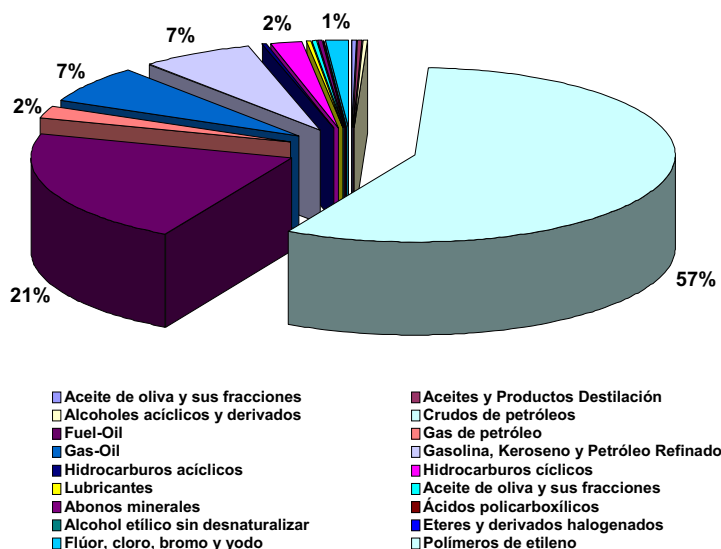
**Figura 1.2.** Áreas de estudio seleccionadas en el golfo de Cádiz (imágenes tomadas de Google™ Earth).

Las fuentes potenciales de contaminación en el entorno de la Bahía de Algeciras son muy diversas, aunque se vinculan a las industrias asentadas en la zona. Asimismo hay que considerar la contaminación difusa y los lixiviados procedentes de la intensa actividad urbana e industrial que se desarrolla en el área, así como una intensa actividad portuaria y tráfico marítimo. Todo ello hace que la Bahía de Algeciras sufra de forma rutinaria vertidos de petróleo, aportes de gases y material particulado a la atmósfera, metales pesados y partículas en suspensión en las aguas, que posteriormente se transfieren a los sedimentos, y en muchos casos alcanzan los suelos de la zona (CSIC, 2003b). La mitad de estos vertidos son urbanos e industriales, un 19 % provienen de las actividades de mantenimiento de los buques como el “bunkering” realizados tanto por parte del puerto de Algeciras como Gibraltar, y un 5 % de la entrada de contaminantes corresponde a vertidos accidentales.

Los aportes urbanos, corresponden fundamentalmente a las aguas residuales de la población asentada en el Campo de Gibraltar. Entre estos núcleos de población cabe destacar: Algeciras, La Línea, San Roque y los Barrios (CSIC, 2003b). Por otro lado, los vertidos industriales de la zona están constituidos por emisiones de gases y material particulado y efluentes líquidos producidos por las industrias situadas en el Campo de Gibraltar, dedicadas fundamentalmente a la producción química: refino de petróleo y petroquímica, así como aquellos asociados a las actividades del tráfico portuario (CSIC, 2003b). Las principales empresas se agrupan en torno a los siguientes núcleos de población:

- Algeciras: Industrias papeleras: Torraspapel, C.L.H y Central Térmica.
- San Roque: Polígono petroquímico: Cepsa, Interquisa, Eastman Chemical, Petresa.
- Los Barrios: Central Térmica, Acerinox, Endesa Puertos.

Organizaciones no gubernamentales, aseguran que alrededor de 4000 y 5000 de los 100000 barcos que cruzan el estrecho cada año, son petroleros, y muchos entran en la bahía de Algeciras a realizar operaciones carga de combustible ("bunkering"). En el caso de Gibraltar, el bunkering se realiza desde un buque – el 'Vemabaltic', capacidad: 107544 Toneladas - fondeado en la misma bahía, y se trata del único lugar de la Unión Europea donde se sigue realizando esta operación desde un buque fondeado (López de Uralde, 2007).



**Figura 1.3.** Mercancías peligrosas cargadas y descargadas en el Puerto Bahía de Algeciras (Martín-Díaz y DelValls, comunicación personal)

Esta evidente influencia antropogénica en la zona y los posibles efectos adversos en el ecosistema de la bahía no han trascendido tanto como por ejemplo el vertido del petrolero *Prestige* en Galicia, aunque cada vez hay más interés y preocupación por los posibles daños de los vertidos en la Bahía de Algeciras.

Otra bahía situada también en el Golfo de Cádiz fue escogida de manera complementaria para llevar a cabo este estudio; una zona que no presenta fuentes de contaminación importantes: la bahía de Cádiz. La Consejería de Medio Ambiente (2007) ha declarado que esta bahía presenta una buena calidad ambiental, y además, el área seleccionada ha sido ampliamente caracterizada y sus sedimentos han sido analizados y evaluados bajo el punto de vista químico y ecotoxicológico resultando ser aptos para su uso como estación de referencia en este trabajo.

### **3. Objetivos e hipótesis**

La hipótesis de partida considera que un ecosistema que recibe de manera continua moderadas dosis de vertidos de hidrocarburos durante un largo periodo de tiempo (impacto crónico) resulta más dañado y presenta mayor polución (contaminación mas efectos) que en el caso de un ecosistema que recibe en un corto periodo de tiempo un vertido de grandes dimensiones (impacto agudo). El objetivo general de esta tesis doctoral consiste en determinar y comparar la calidad de los sedimentos de dos zonas del litoral español afectadas por vertidos de petróleo, la costa gallega y la Bahía de Algeciras frente a una zona considerada no afectada por este tipo de contaminación. Considerando la metodología que se ha seguido, se pretendía realizar esta aportación a través de la consecución de los siguientes objetivos concretos:

1. Determinar el grado de contaminación por los principales contaminantes (metales y PAHs) que se encontraban en los vertidos de hidrocarburos en los sedimentos del Parque Nacional de las Islas Atlánticas y Bahía de Corme-Laxe (costa de Galicia) y en la

desembocadura de los ríos Guadarranque y Palmones en la Bahía de Algeciras así como establecer sus niveles en la Bahía de Cádiz.

2. Establecer el efecto adverso de estos contaminantes una vez que se incorporan a los sedimentos mediante diseño y aplicación de ensayos de laboratorio bajo condiciones controladas y en exposiciones de tipo aguda, letales y bioacumulación utilizando poblaciones de la bacteria *Vibrio fischeri*, de los anfípodos *Corophium volutator* y *Ampelisca brevicornis*, y del poliqueto *Arenicola marina*

3. Caracterizar los posibles efectos adversos de los contaminantes bajo condiciones de laboratorio y campo mediante ensayos de laboratorio crónico y medidas del efecto subletal, que incorporan determinaciones de biomarcadores de exposición (actividad EROD, Metalotioneinas, actividad GST, GPX, GR, FRAP, vitelogenina, TBARS) y de efecto (Alteración de comportamiento, histopatología y daño de ADN) utilizando juveniles de la especie comercial del pez *Sparus aurata* (dorada), el cangrejo *Carcinus maenas*, la almeja *Ruditapes philippinarum* y el poliqueto *Arenicola marina*.

4. Determinación de las alteraciones bentónicas “in situ” que permiten evaluar el posible impacto de los contaminantes sobre el ecosistema marino, a través del estudio de parámetros poblacionales (número de especies, diversidad, riqueza específica, dominancia y presencia de los taxones principales).

5. Identificar las sustancias contaminantes que producen el efecto adverso mediante la integración de los resultados de contaminación (físicoquímicos) y de sus efectos (toxicidad aguda y crónica) bajo condiciones de laboratorio y campo, así como la alteración bentónica,

determinando la calidad de los sedimentos del Parque Nacional de las Islas Atlánticas, la Bahía de Corme-Laxe, y la Bahía de Algeciras, zonas afectadas en mayor o menor medida por vertidos del petróleo.

6. Establecer los niveles de polución en cada una de las zonas estudiadas determinando la severidad de los impactos producidos por cada tipo de vertido estudiados (agudos, vertido Prestige en zona Gallega; crónicos, vertidos continuos Bahía de Algeciras), y siempre por comparación frente a una estación de referencia, localizada en la Bahía de Cádiz.

#### **4. Estructura de la tesis**

Esta tesis doctoral se ha estructurado en seis capítulos: el primero consta de introducción, descripción de los objetivos de la tesis, así como la presentación de las áreas de estudio, mientras que en los cuatro capítulos siguientes se presenta la memoria en sí, finalizando con un último capítulo en el que se muestran las conclusiones obtenidas en el estudio. Cada uno de los cuatro capítulos centrales consta de una introducción y descripción resumida en español y los trabajos de investigación escritos en inglés publicados, aceptados, o bien enviados a distintas revistas internacionales. De esta forma en el capítulo 2 se incluyen los trabajos I, II, III y IV que describen los resultados de toxicidad aguda obtenidos de la realización de los bioensayos con diluciones de fuel extraído del petrolero *Prestige* (trabajos I y II), así como experimentos con muestras ambientales de sedimento (trabajos III y IV). El capítulo 3 presenta los resultados de la evaluación de los efectos subletales bajo condiciones de laboratorio. A lo largo de 5 trabajos se describen los estudios de toxicidad realizados con 4 especies marinas y en los que se han obtenido respuestas subletales de toxicidad tras exponer los organismos a los sedimentos de estudio. El capítulo 4 incluye 4 trabajos en los que se lleva a cabo la evaluación de los

efectos subletales de los contaminantes bajo condiciones de campo. En este ámbito, los trabajos X, XI y XII muestran los resultados de bioensayos realizados con dos especies marinas mediante la instalación en jaulas en los puntos de muestreo, mientras que en el trabajo XIII se comparan las alteraciones bentónicas de las áreas de estudio. En el capítulo 5 se realiza la integración de los resultados mostrados a lo largo de esta memoria, y consta de 3 trabajos. El trabajo XIV realiza un estudio de la evolución de la calidad ambiental de los sedimentos de Galicia a lo largo de los últimos años, mediante el empleo de una metodología TRIAD clásica. En el trabajo XV se desarrolla una nueva metodología dentro de un “weight of evidence approach” en la que se incorpora una nueva línea de evidencia de efectos subletales para la evaluación de la calidad de los sedimentos en el área de Galicia. En el último trabajo, el XVI, se lleva a cabo una integración conjunta de los datos obtenidos en los diferentes estudios de los sedimentos de la zona de Galicia y la Bahía de Algeciras. Finalmente, en el capítulo 6 de esta memoria, se establecen las conclusiones obtenidas tras la consecución de los objetivos propuestos en esta tesis doctoral.

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## Capítulo 2.

### **Análisis de la contaminación y evaluación de la toxicidad aguda mediante ensayos en laboratorio**

La propiedad que tiene una sustancia de producir efectos adversos a un sistema biológico se denomina toxicidad. Sin embargo, el hecho de que una sustancia tóxica esté presente en el medio no implica necesariamente que vaya a causar efectos tóxicos. Una sustancia presente en el medio ambiente a concentraciones superiores de las naturales conlleva un episodio de contaminación, y en el caso de que también presente efectos tóxicos sobre la biota estaremos hablando del fenómeno de polución. En este último caso, dosis bajas de contaminante pueden producir alteraciones en las funciones vitales de los organismos, mientras que dosis altas de la misma sustancia pueden ser letales.

Tradicionalmente se ha evaluado la calidad ambiental de un sedimento mediante el análisis de las concentraciones de contaminantes y la comparación con guías numéricas (SQGs). De este modo se pretendía evaluar el riesgo potencial de los contaminantes asociados al sedimento (Casado-Martínez, 2006). En la actualidad se han propuesto determinaciones basadas en análisis químicos junto con ensayos de toxicidad en laboratorio con el fin de llevar a cabo un estudio de los procesos de polución en ecosistemas costeros. Los bioensayos de toxicidad son instrumentos que se emplean para determinar la

ecotoxicidad y biodisponibilidad que producen los compuestos químicos del sedimento en los organismos bentónicos. En este tipo de experimentos, los organismos se exponen a muestras de sedimento y tras un periodo de exposición se mide una respuesta biológica. Esta respuesta ha de ser sensible, relevante y fácil de estandarizar (Stebbing et al., 1980).

La toxicidad aguda hace referencia al efecto nocivo resultante de una exposición relativamente corta a una sustancia tóxica. Estos efectos sobre el organismo suelen desarrollarse rápidamente y suelen dejar de aparecer en el momento que cesa la dosis. Los ensayos de toxicidad aguda se realizan en un periodo de tiempo que puede variar de minutos a varios días, y van a proporcionar respuestas puntuales (mortalidad, inhibición del crecimiento, inhibición de la bioluminiscencia, etc.)

En este capítulo se presentan cuatro trabajos en los que se llevan a cabo distintos ensayos de toxicidad aguda con cuatro especies de invertebrados marinos. En los primeros dos trabajos (I y II) se lleva a cabo un estudio preliminar para determinar la toxicidad del fuel del petrolero *Prestige*. Para ello se realizaron una serie de diluciones de fuel extraído del barco hundido con sedimento limpio procedente de la Bahía de Cádiz, de forma que se obtuvieron distintas concentraciones de fuel en sedimento. En el trabajo I se efectuaron exposiciones con el anfípodo *Ampelisca brevicornis* y tras un periodo de diez días se contabilizó la mortalidad en cada una de las diluciones. Los resultados obtenidos permitieron llevar a cabo el cálculo del LC50, parámetro toxicológico que indica la concentración de sustancia capaz de producir una mortalidad del 50 %. Los anfípodos son utilizados de manera habitual en tests de toxicidad y se correlacionan positivamente con cambios en las comunidades bentónicas.

En el segundo trabajo (II) se llevó a cabo una exposición similar aunque esta vez con el poliqueto *Arenicola marina* y se calculó el LC50 tras 10 días de

exposición y un segundo LC50 pasados 21 días; además se determinó el factor de bioacumulación (BCF) para PAHs a partir de la relación entre la concentración de contaminante en el organismo frente a la concentración del mismo en el sedimento. El poliqueto *Arenicola marina* mostró ser menos sensible frente a la contaminación orgánica que el anfípodo *Ampelisca brevicornis* aunque a su vez presenta la capacidad de habitar en zonas polucionadas ofreciendo la posibilidad de llevar a cabo estudios de bioacumulación.

**Tabla 2.1.** Relación de bioensayos agudos realizados para la evaluación de la calidad de los sedimentos (adaptado de Casado-Martínez et al., 2006).

Bioensayo	Especie	Medida final	Ruta de exposición	Tiempo de exposición
Microtox®	<i>Vibrio fischeri</i>	Inhibición de la bioluminescencia (IC50)	Lixiviado	5 - 30 minutos
Anfípodos	<i>Ampelisca brevicornis</i>	supervivencia	Fase sólida	10 días
Anfípodos	<i>Corophium volutator</i>	supervivencia	Fase sólida	10 días
Poliquetos	<i>Arenicola marina</i>	Supervivencia y bioacumulación	Fase sólida	10 – 15 días

El tercer trabajo (III) muestra la primera aproximación al estudio de los sedimentos de la Bahía de Algeciras y el Parque Nacional de las Islas Atlánticas en Galicia. 14 puntos de muestreos fueron evaluados junto a dos controles (negativo y positivo) mediante el uso de una caracterización físico química de los sedimentos y su relación con resultados de dos ensayos de toxicidad: Microtox® y el test de 10 días con el anfípodo *Corophium volutator*. El test Microtox® se basa en la medición de la emisión de luz de una bacteria marina *Vibrio fischeri*, la cual se expone a una serie de diluciones de una muestra de sedimento. La bioluminiscencia es directamente proporcional al estado metabólico de la célula; cuando la bacteria se expone a una sustancia tóxica, el

cuerpo celular sufre algunos cambios de forma que la bioluminiscencia emitida disminuye. El test evalúa la disminución de la bioluminiscencia después de la exposición de los organismos al sedimento, de forma que los resultados obtenidos se expresan en términos de la concentración efectiva de una determinada sustancia presente en el medio que produce una reducción de la emisión del luz del microorganismo del 50% (IC50). La Normativa Canadiense establece que una muestra es tóxica cuando el valor de IC50 es menor de 1000 mg L<sup>-1</sup> peso seco y no tóxica cuando el medido es mayor de 1000 mg L<sup>-1</sup> peso seco. Por su parte, el Centro de Estudios y Experimentación de Obras Públicas (CEDEX) define una muestra como tóxica cuando IC50 es menor de 750mg L<sup>-1</sup> peso seco y no tóxica cuando éste es mayor de 750 mg L<sup>-1</sup>. También existen distintos criterios para considerar a una muestra como tóxica en función del resultado obtenido en el test agudo realizado con anfípodos. Todos estos criterios utilizan el valor del porcentaje de mortalidad de anfípodos que se obtiene restando a 100 el valor del porcentaje de supervivencia observado. En este sentido son diversos los países que incluyen este test dentro de las baterías para establecer la nocividad de muestras de sedimentos y/o material de dragado. Entre ellos cabe destacar la Normativa Holandesa, establecida por el Ministerio de fomento Holandés que considera que una muestra de sedimento es tóxica si la mortalidad de la especie *Corophium volutator* es igual o mayor del 25%. Por su parte, la Normativa Inglesa considera que, para esta misma especie de anfípodo, la mortalidad de la población expuesta ha de ser igual o mayor al 40% para que la muestra de sedimento sea considerada tóxica. La Normativa para el área de Hong Kong utiliza una especie de anfípodos no detallada en los test de toxicidad, y propone que la mortalidad de los individuos ha de ser igual o mayor al 30% para considerar que existe toxicidad. Por otra parte, existen normativas donde no se proponen un único valor indicativo sino que se utiliza un doble criterio, incluyendo un valor observado y un criterio estadístico. En este sentido, la Normativa Estadounidense, elaborada por la Environmental

Protection Agency (USEPA) y el cuerpo de ingenieros del Ejército Americano, utiliza la especie *Ampelisca abdita* y establece como criterio de toxicidad una mortalidad superior al 20% con respecto al sedimento de referencia y además significativamente diferente ( $p < 0.05$ ) del control. El Centro de Estudios y Experimentación de Obras Públicas (CEDEX) propone este mismo criterio doble para clasificar las muestras de sedimento como tóxicas o no tóxicas para el litoral español. Los resultados de este trabajo muestran las diferencias entre ambas zonas de estudio y determina que la Bahía de Algeciras presenta una degradación ambiental mayor que aquella observada en las costas gallegas.

En el último trabajo (IV) de este segundo capítulo, se aplican los test de toxicidad anteriormente descritos: anfípodos, Microtox® y *Arenicola marina* para evaluar la toxicidad de sedimentos muestreados en las costas gallegas cuatro años después del vertido del petrolero *Prestige*. Parte de este estudio, en concreto los análisis de bioacumulación de PAHs en el poliqueto *A. marina*, se realizaron durante una estancia en el centro IPIMAR en Lisboa. Los resultados indican una disminución de la contaminación con respecto a estudios previos, así como una desaparición de la toxicidad aguda aunque una importante bioacumulación de PAHs fue detectada principalmente en el área de Corme-Laxe, lo que sugiere que a pesar de que la calidad ambiental se ha recuperado de forma notable, existe la posibilidad de efectos subletales en la biota.

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# Acute toxicity of residual fuel oil from the tanker “Prestige” using amphipods

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## Abstract

In November 2002, the simple hull tanker “Prestige” sank in the Galician Coast (NW Spain) and spilt 63,000 Tons of fuel oil. Two years after the spill, the remaining oil has been extracted from the hull and has been physicochemically and ecotoxicologically characterized. An acute bioassay using the amphipod *Ampelisca brevicornis* has been carried out in order to determine toxicity associated with the contaminants presents in the fuel. The bioassay was conducted by exposing during 10 days the individuals of *Ampelisca* to clean sediment mixed with different proportions of fuel oil (0.1%, 0.5%, 2%, 8%, 16% and 32%). Results were linked with the chemical data in order to determine the sensitivity of the amphipod to the fuel oil compounds. The LC50 value obtained for *Ampelisca brevicornis* ( $1.37\% \pm 0.33$ ) and the PAHs concentrations measured in the fuel oil have permitted to calculate the Sediment Quality Values (SQVs) that are similar from those obtained from previous studies corroborating that the acute toxicity of the fuel oil it was mainly associated with the concentration of PAHs.

**Keywords:** PAHs, *Ampelisca brevicornis*, quality values, bioassay, sediment toxicity, sediment dilution.

## 1. Introduction

On 13th November 2002 the tanker *Prestige* broke down in the Galician Coast (NW Spain) and sank six days later in water 3,500 metres deep. In all, it is estimated that the *Prestige* spilt 63,000 tonnes of heavy fuel oil leading to one of the greatest ecological catastrophes in Spain. On September 2004 the 58,000 tonnes of remaining fuel that were still in the tanker were finally collected.

The composition of this fuel was a mixture of saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes, being most of the polycyclic aromatic hydrocarbons -PAHs- of an intermedium-high molecular weight (Blanco et al., in press). The physicochemical characteristics of the oil spilled by the tanker *Prestige* show that the soluble fraction is low and the kinetic of degradation is slow under natural conditions so it is expected to be accumulated in sediments (CSIC 2003). The biological effects associated with the chemicals from the oil spill will be dependent on the nature of the ecosystem that accepts them and the organisms living in it (DelValls 2003). The first research notes about the early impact support that the acute toxicity of the weathered fuel (Mariño-Balsa 2003), very rich in high molecular weight compounds, was relatively low for the organisms tested (clams and microalga). The Polycyclic aromatic hydrocarbons (PAHs) with intermediate to high molecular weight do not usually show severe toxicity within their solubility limits in water. However, it is necessary to note that some PAHs can become more dangerous due to their photomodification. This is particularly dangerous for organisms living in the intertidal zone or near the water surface (Carballeira 2003). Reported responses of infauna after an oil spill include very high initial mortalities in species sensitive to hydrocarbons, such as crustaceans and especially amphipods, and their subsequent disappearance (Pearson et al. 1978; Sanders et al. 1980; Glémarec and Hussenot 1982; Gray and Pearson 1982).

Sediment toxicity tests provide information on the toxicity of contaminated sediments that can be neither derived from chemical analysis nor from ecological surveys performed alone (Chapman and Long 1983; Long and Chapman 1985). The species of organisms used in the sediment toxicity tests should provide an appropriate indication of the hazards of chemical stressors in the sediment (Chapman et al. 2002). Amphipods are generally acknowledged as the organism's choice for many sediment toxicity assessments, and amphipod toxicity test results can correlate positively with changes in benthic communities (Long et al. 2001; Marín-Guirao et al. 2005).

The aim of this work is to assess the sensibility of the amphipod *Ampelisca brevicornis* to the contamination associated with the remanent fuel oil collected from the tanker Prestige on September 2004 and to establish the usefulness of the toxic response measured for further management of sediments contaminated by the oil spill and in general by organic pollutants. In order to reach these objectives, a bioassay was conducted exposing a population of the amphipod *Ampelisca brevicornis* to different dilutions of fuel oil with clean sediment.

## **2. Material and methods**

### **2.1. Approach**

The present study was carried out using six different sediment dilutions of fuel oil. The oil used was extracted from the remaining fuel of the tanker *Prestige* (September 2004) and was mixed with clean sediment from the Bay of Cádiz (0.1%, 0.5%, 2%, 8%, 16% and 32 % - dry weight of fuel oil-) that was also used as negative control (BC). Clean sediment was collected in a pristine area of the Bay of Cádiz (Riba et al. 2003) and was filtered (0.6 mm) prior to the toxicity test in order to remove means interferences as shells, predators and other

residues. These sediments were dried and homogenized at room temperature prior to chemical analysis.

Individuals of the specie *Ampelisca brevicornis* used in the bioassay were collected from the clean sediment (negative control) located in the intermareal zone of the Bay of Cádiz, by sieving the sediment through a 0.6 mm mesh, as reported by Riba et al. (2003). They were immediately transported to the laboratory where they were placed in 11 liters aquariums with clean seawater and sieved sediment from the same location. Aeration was provided and natural photoperiod was selected. During acclimatation the organisms were fed twice a week with a special food for invertebrates (mixture made of aminoacids and organic particles) and water was replaced.

## 2.2. Chemical analysis of sediments

For trace metal analysis (Ni, V, Cd, Pb, Cr, Co) the sediment was digested as described by Loring and Rantala (1992). Trace metals were measured by graphite furnace atomic absorption spectrophotometry (Perkin-Elmer 4100 ZL) (Cobelo-García et al. 2005) Results are expressed as  $\text{mgkg}^{-1}$  dry sediment. The analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) and showed agreement with the certified values higher than 90%.

Polycyclic aromatic hydrocarbons (Fluorene, Acenaphthene, Naphthalene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo[a]anthracene, Chrysene, Benzo[a]fluoranthene, Benzo[e]pyrene, Benzo[a]pyrene, Perilene, Dibenzo[ah]anthracene, Indene[123-cd]pyrene, Benzo[ghi]perilene) were analyzed by using a gas chromatography equipped with an electron capture detector (GC/MS) (U.S. Environmental Protection Agency SW-846 Method 8270) (USEPA 1984). Briefly, dried samples were soxhlet extracted with *n*-hexane for 18 h, and the extracts were isolated by column chromatography on Florisil-alumino-silica. PAHs were eluted and their

fractions were dried in a rotatory evaporator and re-dissolved in isooctane. Aromatic fractions were analyzed on a Hewlett–Pakard (HP) 5890 Series II gas chromatograph coupled with HP 5970 mass spectrometer. Chromatographic resolution was achieved with a 30 m × 0.250 mm DB-5 capillary column, which has a 0.25 µm film thickness, with helium as carrier gas. Quality control was carried out using NRC-CNRC HS-6 sediment reference material. The analytical procedure allow agreement with the certified values higher than 90%.

### 2.3. Toxicity test

The toxicity test was performed exposing individuals of the amphipods *Ampelisca brevicornis* to bulk sediment using the percentage of survival after ten days of exposure as the end point (ASTM 1993). The dilutions with clean sediment and fuel oil (0.1%, 0.5%, 2%, 8%, 16% and 32% -dry weight of fuel oil-) and the negative control (200 g) were placed in 2 L glass beakers and about 800 mL of clean seawater were added. When the sediment settled down in the beakers, aeration was provided, and 12 hours after the individuals were sieved from the acclimatization aquariums and 20 adults (3-5 mm) of *Ampelisca* where placed in each replicate. No food was provided during the experiment. The containers where kept in an incubator with photoperiod 12h-light/12h-dark and maintained at  $19 \pm 1$  °C during the 10 days of exposure. After that time, the beakers where sieved and the survival was counted in each replicate.

### 2.4. Data calculation

The mortality of *Ampelisca brevicornis* measured after 10 days of exposure time was used to derive a toxic parameter (LC50) associated with the fuel oil. From the toxic responses (mortality) obtained during the exposure to the different dilutions (0.1%, 0.5%, 2%, 8%, 16% and 32%) was defined the concentration (percentage of dry weight of fuel oil) that provokes the mortality

of the 50% of the *Ampelisca brevicornis* population exposed. The LC50 was calculated by linear regressions of log toxicant dilution of fuel oil on declining probit values (probit-analysis-program, version 1.5).

Sediment quality values (SQVs) have been calculated in order to identify the concentration of PAHs (Fluorene, Acenaphthene, Naphthalene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo[a]anthracene, Chrysene, Benzofluoranthene, Benzo[e]pyrene, Benzo[a]pyrene, Perilene, Dibenzo[ah]anthracene, Indene[123-cd]pyrene, Benzo[ghi]perilene) responsible of the toxicity associated with the fuel oil from the *Prestige* spill. The SQVs have been calculated using the LC50 values obtained and the concentration of individuals PAHs measured in the fuel oil. Thus, the SQVs are defined as the concentration of individual and total PAHs associated with the mortality of 50% of the total population of amphipods after 10 days of exposure to fuel oil.

### 3. Results

Table 1 shows summarized results of contaminants -total and individual PAHs and trace metals (Ni, V, Cd, Pb, Cr, Co) expressed as mg Kg<sup>-1</sup> dry sediment- that were measured in the dilutions of fuel oil (0.1%, 0.5%, 2%, 8%, 16% and 32% -dry weight of fuel oil-) and in the negative control (BC).

The pure fuel oil extracted from the tanker *Prestige* (September 2004) presents a concentration of total PAHs of 1443 mg Kg<sup>-1</sup> -dry weight-, whereas in the negative control (BC) PAHs were not detected; therefore, the concentration of PAHs in the dilutions depends only in the presence of PAHs in the fuel oil. Regarding to the content of trace metals in the fuel oil results show that levels of these contaminants are not high except for Ni (55 mg kg<sup>-1</sup>) and V (170mg kg<sup>-1</sup>).

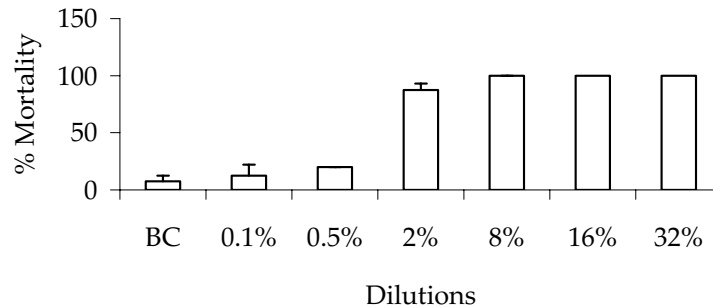
It has been observed that the individual PAHs predominant in the fuel oil are Naphthalene (395 mg kg<sup>-1</sup>), Phenanthrene (385 mg kg<sup>-1</sup>), Pyrene (111 mg kg<sup>-1</sup>), Chrysene (mg kg<sup>-1</sup>), Fluorene (99.3 mg kg<sup>-1</sup>) and Acenaphthene (75.3 mg kg<sup>-1</sup>).

**Table 1.** Total PAHs and metal concentration in mg Kg<sup>-1</sup> -dry sediment- measured in the negative control and in the fuel oil dilutions.

		Oil	BC	0.10%	0.50%	2%	8%	16%	32%
<b>PAHs</b>	<b>Total PAHs</b>	1443	n.d	0.72	3.61	14.4	57.7	115	231
	<b>Fluorene</b>	99.3	n.d	0.05	0.25	0.99	3.97	7.95	15.9
	<b>Acenaphthene</b>	75.3	n.d	0.04	0.19	0.75	3.01	6.03	12.1
	<b>Naphthalene</b>	395	n.d	0.20	0.99	3.95	15.8	31.6	63.2
	<b>Phenanthrene</b>	385	n.d	0.19	0.96	3.85	15.4	30.8	61.6
	<b>Anthracene</b>	51.4	n.d	0.03	0.13	0.51	2.05	4.11	8.22
	<b>Fluoranthene</b>	28.5	n.d	0.01	0.07	0.29	1.14	2.28	4.57
	<b>Pyrene</b>	111	n.d	0.06	0.28	1.11	4.43	8.86	17.7
	<b>Benzo[a]anthracene</b>	55.9	n.d	0.03	0.14	0.56	2.24	4.48	8.95
	<b>Chrysene</b>	102	n.d	0.05	0.22	9.98	3.63	7.12	14.3
	<b>Benzo[fluoranthene]</b>	16.0	n.d	0.01	0.04	0.16	0.64	1.28	2.56
	<b>Benzo[e]pyrene</b>	45.7	n.d	0.02	0.11	0.46	1.83	3.65	7.31
	<b>Benzo[a]pyrene</b>	29.7	n.d	0.01	0.07	0.30	1.19	2.37	4.75
	<b>Perilene</b>	11.4	n.d	0.01	0.03	0.11	0.46	0.91	1.83
	<b>Dibenzo[ah]anthracene</b>	5.70	n.d	0.00	0.01	0.06	0.23	0.46	0.91
	<b>Indene[123-cd]pyrene</b>	5.23	n.d	0.00	0.01	0.04	0.16	0.32	0.65
	<b>Benzo[ghi]perilene</b>	17.1	n.d	0.01	0.04	0.17	0.68	1.37	2.74
<b>Metals</b>	<b>Ni</b>	55	14.1	7.07	7.15	7.46	8.69	10.3	13.6
	<b>V</b>	170	80.0	40.1	40.2	40.9	43.6	47.2	54.4
	<b>Cd</b>	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	<b>Pb</b>	n.d	23.0	11.5	11.4	11.3	10.6	9.7	7.80
	<b>Cr</b>	0.31	31.0	15.5	15.4	15.2	14.3	13.0	10.6
	<b>Co</b>	n.d	3.40	1.70	1.69	1.67	1.56	1.43	1.16

Figure 1 shows summarized results of the amphipods mortality (%) after 10 days of exposure to the different dilutions of fuel oil. Mean survival in all the replicates of clean sediment from the Bay of Cádiz (control) was higher than 95%. The mortality of *Ampelisca* increases with the % of the fuel oil in the dilutions. Results obtained at the lowest dilution of fuel oil (0.1%) show a survival ranged between 80-100% during the ten days of exposure. All

replicates in the concentration of 0.5% of fuel oil indicate a survival of 80% in this dilution. The mortality registered for the dilution of 2% of fuel oil ranged between 70-100% whereas no survival was detected in the highest dilutions of fuel oil (8%, 16 % and 32%) after the exposure period.



**Figure 1.** Average and standard deviations of the percentage of mortality of the amphipod *Ampelisca brevicornis* after 10 days of exposure to each dilution of fuel oil.

From the mortality obtained during the exposure of the amphipods to the different dilutions it has been calculated the percentage of dry weight of fuel oil that provokes the mortality of the 50% of the *Ampelisca brevicornis* population exposed. The lethal concentration (LC50) of dry weight of fuel oil associated with the toxic responses calculated in *Ampelisca brevicornis* is  $1.37 \pm 0.33$  (percentage of dry weight of fuel oil).

These LC50 value obtained for *Ampelisca brevicornis* and the PAHs concentrations measured in the fuel oil and in the negative control (BC) have permitted to calculate the Sediment Quality Values (SQVs) shown in table 2. The SQVs obtained for the different contaminants are compared to other values

**Table 2.** Sediment quality values for PAHs ( $\mu\text{g kg}^{-1}$  –dry weight-) are obtained from the LC50 and calculated for the *Ampelisca brevicornis* used in the sediment toxicity test. Sediment Quality Guidelines were derived using previous studies data (ERL= Effects Range-Low and ERM= Effects Range-Median, NOAA (1999); Puget Sound AET, Swartz et al. (1985, 1986); Commencement Bay, Tetra Tech (1985); San Francisco Bay, Long et al. (1989)).

PAHs ( $\mu\text{g kg}^{-1}$ )	<i>Ampelisca brevicornis</i>	ERL	ERM	Puget Sound AET	Commencement Bay	San Francisco Bay
Total PAHs	19790.7	4022	44792	11752±14548	16771	n.a.
Fluorene	1362	19	540	1000	707±1341	n.a.
Acenaphthene	1033	16	500	2000	654±1049	n.a.
Naphthalene	5417	160	2100	2400	1564±1735	n.a.
Phenanthrene	5276	240	1500	5400	2838±4603	510
Anthracene	705	85	1100	1900	476.2±549.2	1100
Fluoranthene	391	600	5100	n.a.	n.a.	n.a.
Pyrene	1519	665	2600	4300	1820±2252	2600
Benzo[a]anthracene	767	261	1600	1600	931±1322.8	1100
Chrysene	1398	384	2800	2800	1363±1970	2100
Benzo[fluoranthene	219	n.a.	n.a.	n.a.	n.a.	n.a.
Benzo[el]pyrene	626	n.a.	n.a.	n.a.	n.a.	n.a.
Benzo[al]pyrene	407	430	1600	2400	890±1322	432±344
Perilene	157	n.a.	n.a.	n.a.	n.a.	n.a.
Dibenzol[ah]anthracene	78	63	260	260	183±344	80±88
Indene[123-cd]pyrene	71.7	n.a.	n.a.	n.a.	n.a.	n.a.
Benzo[ghi]perilene	235	n.a.	n.a.	n.a.	n.a.	n.a.
Ni	7.3	20.9	51.6	n.a.	41±32	99±35
V	40.6	n.a.	n.a.	n.a.	n.a.	n.a.
Cd	0.0	1.2	9.6	93	63.2±148	70
Pb	11.3	46.7	218	660	170.8±192	95.7±93
Cr	15.3	81	370	n.a.	n.a.	141.8±86.5
Co	1.7	n.a.	n.a.	n.a.	n.a.	n.a.

proposed by previous studies (NOAA 1999) which show a compilation of Sediment Quality Guidelines that have been calculated based on a wide ranged data base (Long and Morgan 1991; Long et al. 1995). NOAA (1999) explains the 10th percentile values named the ERL (Effects Range-Low) as the concentrations below which adverse effects rarely occur, whereas the 50th percentiles named ERM (Effects Range-Median) values, are representative of concentrations above which effects frequently occur (NOAA 1999). Some examples from SQVs obtained from previous studies (table 2) with amphipods - Puget Sound AET, Commencement Bay and San Francisco Bay- (Long and Morgan 1991) are similar to those recorded with the present study for *A. brevicornis*.

#### 4. Discussion

Results obtained from the chemical analysis show a content of total PAHs of 1443 mg Kg<sup>-1</sup> -dry weight- from which more than 27% is Naphtalene (395 mg Kg<sup>-1</sup> -dry weight-). Previous studies (Albaigés and Bayona 2003) show that Naphtalene may significantly accumulate in the biota and that concentrations of Naphtalene in sediments higher than 34.6 mg Kg<sup>-1</sup> -dry weight-) may produce negative effects on the benthic organisms. Specifically, oils spills such as the *Sea Empress* have involved a decline in the amphipod fauna with the genera *Ampelisca* particularly affected (Nikitik and Robinson 2003). The LC50 obtained from the acute bioassay using *Ampelisca brevicornis* shows that the fuel oil is toxic at concentrations equal or lower than 1.37%. This value and the concentration of chemicals on the fuel oil and clean sediment, allows calculating the Sediment Quality Values (SQVs) for each contaminant.

Results of SQVs for metals are lower than the NOAA guidelines what explains that the toxicity it is not probably associated with the concentration of

metals in the fuel oil dilutions. On the other hand PAHs seem to be responsible for the acute toxicity measured at the end of the test and the SQVs obtained for total PAHs ( $19790.7 \mu\text{g kg}^{-1}$ ) are in the same range than those previously reported for these contaminants by different authors ( $4022 \mu\text{g kg}^{-1}$  (ERL) –  $44792 \mu\text{g kg}^{-1}$  (ERM) (NOAA 1999);  $11752 \pm 14548 \mu\text{g kg}^{-1}$  (Puget Sound AET) (Swartz et al. 1985; Swartz et al. 1986);  $16771 \mu\text{g kg}^{-1}$  (Commencement Bay) (Tetra Tech 1985)). Most of the SQVs of individuals PAHs calculated in the present study surpass the ranges or they are in the same range as the SQGs recorded in previous studies (NOAA 1999; Swartz et al. 1985; Swartz et al. 1986; Tetra Tech 1985; Long and Buchman 1989). Naphtalene shows the highest value of SQV of the total PAHs ( $5417 \mu\text{g kg}^{-1}$ ) which is higher than the data recorded in previous studies ( $2400 \mu\text{g kg}^{-1}$  (Puget Sound AET);  $1564 \pm 1735 \mu\text{g kg}^{-1}$  (Commencement Bay);  $160 \mu\text{g kg}^{-1}$  (ERL) -  $2100 \mu\text{g kg}^{-1}$  (ERM)(NOAA 1999)). The SQV calculated from the *A. brevicornis* toxicity test for Fluorene ( $1362 \mu\text{g kg}^{-1}$ ), is also higher than those SQGs from other studies as it is shown in table 2, but it is closed to the value calculated for Commencement Bay ( $707 \pm 1341 \mu\text{g kg}^{-1}$ ) and Puget Sound AET ( $1000 \mu\text{g kg}^{-1}$ ) for this individual PAH. Acenaphthene presents a SQV ( $1033 \mu\text{g kg}^{-1}$ ) closed to the SQG for Commencement Bay ( $654 \pm 1049 \mu\text{g kg}^{-1}$ ). The SQV calculated for the individual PAH Phenanthrene ( $5276 \mu\text{g kg}^{-1}$ ) is closed to those SQGs from Commencement Bay ( $2838 \pm 46031 \mu\text{g kg}^{-1}$ ) and Puget Sound AET ( $5400 \mu\text{g kg}^{-1}$ ) whereas the SQV for Anthracene derived from the *A. brevicornis* toxicity test ( $705 \mu\text{g kg}^{-1}$ ) is in the range of the same values proposed by NOAA (1999) ( $85 \mu\text{g kg}^{-1}$  (ERL) -  $1100 \mu\text{g kg}^{-1}$ ) and it is closed to the SQG derived for San Francisco Bay ( $1100 \mu\text{g kg}^{-1}$ ). Similar comparisons are for SQVs of Pyrene ( $1519 \mu\text{g kg}^{-1}$ ), Benzo[a]anthracene ( $767 \mu\text{g kg}^{-1}$ ) and Dibenzo[ah]anthracene ( $78 \mu\text{g kg}^{-1}$ ) which are in the range of NOAA (1999) ( $665 \mu\text{g kg}^{-1}$  (ERL) -  $2600 \mu\text{g kg}^{-1}$ ;  $261 \mu\text{g kg}^{-1}$  (ERL) -  $1600 \mu\text{g kg}^{-1}$ ;  $63 \mu\text{g kg}^{-1}$  (ERL) -

260  $\mu\text{g kg}^{-1}$ , respectively), whereas Benzo[a]anthracene is also closed to the SQG in San Francisco Bay (1100  $\mu\text{g kg}^{-1}$ ). The SQV obtained for Chrysene (1519  $\mu\text{g kg}^{-1}$ ) is quite closed to the SQG determined for Commencement Bay (1363 $\pm$ 1970  $\mu\text{g kg}^{-1}$ ) and is in the range determined by NOAA (1999) (384  $\mu\text{g kg}^{-1}$  (ERL) - 2800  $\mu\text{g kg}^{-1}$ ). The data obtained for Fluoranthene (391  $\mu\text{g kg}^{-1}$ ) and Benzo[a]pyrene (407  $\mu\text{g kg}^{-1}$ ) are lower than those obtained in previous studies (table 2), although the SQV of Benzo[a]pyrene is closed to the ERL of NOAA (1999) (430  $\mu\text{g kg}^{-1}$ ).

From the comparisons carried out, we can say that the SQVs obtained from the *A. brevicornis* acute toxicity test, are similar to those reported in previous studies for the same contaminants. This corroborates the fact that the toxicity measured in the test is due to the presence of PAHs in the fuel oil. Benzo[a]fluoranthene, Perilene, Dibenzo[a,h]anthracene, Indene[123-cd]pyrene and Benzo[ghi]perilene show the lowest SQVs obtained from the experiment what means that these PAHs are potentially the most toxic agreeing with other studies (NOAA 1999; Swartz et al. 1985; Swartz et al. 1986; Tetra Tech 1985; Long and Buchman 1989; Batelle 2000; Lee et al. 2001). However these higher molecular weight PAHs probably are tightly bound to particles what decreases their bioavailability.

Further studies would be need to establish the toxicity effect of each of the individual PAHs presented in the fuel oil of the tanker *Prestige*, specially when they reach the environment where they can suffer changes in the proportion of individuals PAHs and become more dangerous due to photomodification (DeValls 2003; Carballeira 2003). Previous studies (Pelletier et al. 1997; Pelletier et al. 2000) have shown that Anthracene, Fluoranthene and Pyrene increase their toxicity on larvae and embryos of marine invertebrates. In this sense, studies carried out with environmental sediment samples affected by

the oil spill will be able to establish the toxicological effect of the individuals PAHs once they have been modified by the environmental conditions being the work here presented a first step to address the potential adverse effects of the contaminants presented in the original fuel when diluted with natural and clean littoral sediments.

## **5. Conclusions**

Previous studies (Riba et al. 2003) have shown that the amphipod specie *Ampelisca brevicornis* is a sensitive organism valid to assess toxicity of sediments contaminated by trace metals. Results obtained in this study show that this specie can be also used to assess sediment toxicity associated with samples contaminated by PAHs, and sediment bioassays with *Ampelisca brevicornis* could be included in the assessment of oil spills to determine acute toxicity responses and to derive Sediment Quality Guidelines.

In this sense, the amphipod *Ampelisca brevicornis* has been validated as an appropriate organism to conduct sediment toxicity tests with metals and hydrocarbons contaminated sediments and makes this specie suitable for the assessment of hazardous materials that may accumulate in sediments and produce effects on the biota.

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## Using the polychaete *Arenicola marina* to determine toxicity and bioaccumulation of PAHS bound to sediments

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**Abstract** The present study was conducted to evaluate a sediment toxicity and bioavailability test with the polychaete *Arenicola marina* as a potential tool to assess sediments contaminated by oil spills. A bioassay using the lugworm *Arenicola marina* was carried out in order to determine toxicity and bioaccumulation associated with the contaminants present in the fuel oil extracted from a sank tanker. After 10 and 21 days of exposure to sediments with different proportions of fuel oil (0.5, 1, 2, 4 and 8%) polychaetes were sampled to determine the mortality and the levels of individual PAHs in the organisms. During the experiment, mortality was recorded and the concentration (percentage of fuel oil) that provokes the mortality of the 50% of the *Arenicola marina* population exposed was

calculated for both sampling dates (LC50(10)=6.4%; LC50(21)=2.4%). Bioaccumulation was mainly produced for fluoranthene, pyrene, benzo(b)fluoranthene and benzo(k)fluoranthene, whereas phenanthrene and anthracene were initially accumulated and then metabolized. The results obtained in the present study suggest *Arenicola marina* can be a suitable species for assessing PAHs toxicity and bioaccumulation as part of oil spill management.

**Keywords** LC50 · Quality values · Sediment toxicity · Oil spill

### Introduction

The biological effects associated with the chemicals from the oil spill depend on the nature of the affected ecosystem (DelValls 2003). Petroleum can adversely affect organisms by physical action (smothering, reduced light), habitat modification (altered pH, decreased dissolved oxygen, decreased food availability) and toxic action (Albers 2003). The remaining fuel from an important oil spill in the North of Spain (*Prestige*, 2002) that was still in the tanker was eventually extracted in 2004; the composition of this heavy fuel-oil (type M-100) was a mixture of saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes, being most of the polycyclic aromatic hydrocarbons – PAHs – of an intermedium-

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high molecular weight (Alzaga et al. 2004; Blanco et al. 2006). PAHs are a ubiquitous group of contaminants and can accumulate and persist in marine sediments (Neff 1979), and affect organisms through toxic action (Albers 2003).

Sediment bioassays (toxicity and bioaccumulation) are instruments used to test the toxicity and bioavailability of chemical compounds in sediments to benthic organisms. In the present study we selected *Arenicola marina*, in order to test the toxicity and bioaccumulation of the fuel oil extracted from the tanker. *A. marina* is a bulk sediment feeding polychaete worm that lives in a U-shaped burrow. This species was chosen for the bioassay because it: (a) is continuously exposed to contaminants in the sediment, which it ingest while feeding; (b) is available all the year round, often in reasonably high densities; (c) tolerates a wide range of particle sizes and salinities, (d) has a broad geographic range; and (e) supposes an important species in coastal food chain (Bat and Raffaelli 1997). In addition this species has been recommended by Oslo-Paris Commission (1995) as monitoring organism.

Accumulation of hydrophobic organic contaminants by benthic organisms can occur either from aqueous phase or dietary exposure (Lamoureux and Brownawell 1998). Uptake of hydrophobic compounds from ingested material has been reported as a major contributor to an animal's total body burden of toxicants (Kaag et al. 1996; Kaag et al. 1998; Penry and Weston 1998; Selck et al. 2003). Also bioaccumulation studies have shown that lugworms accumulate organic contaminants to higher concentrations than filter feeding animals (Kaag et al. 1997).

The ability of organisms to metabolize and excrete PAHs also has been shown to be related to bioaccumulation (Rust et al. 2004a, 2004b), where species with limited metabolic ability tend to accumulate higher PAHs concentrations in their tissue (Varanari et al. 1985; Driscoll and McElroy 1996; Rust et al. 2004b). Even though the presence of a PAH metabolising system in *A. marina* (Christensen et al. 2002) has been strongly suggested, invertebrates tend to excrete metabolites more slowly than vertebrate species (Rust et al. 2004b). Metabolites have been found to be eliminated at rates either greater or lower than those of the parent compound (Spacie and Hamelink 1995). Therefore, toxicity will depend on a combination of relative retention time and relative toxicity of

parent versus metabolites (Selck et al. 2003). Previous studies agree that *A. marina* appears to be an appropriate choice as indicator species for PAH bioaccumulation (Rust et al. 2004b), and a suitable organism to monitor PAH pollution.

The aim of this study is to assess the sensitivity of the polychaete *A. marina* to the contamination associated with PAHs from oil spills by using the remaining fuel oil extracted from a tanker and to determine the bioavailability of PAHs present in the fuel oil by measuring the bioaccumulation in the exposed organisms. The results obtained will permit the calculation of the Sediment Quality Guidelines (SQGs) for this group of PAHs and will help to predict toxicity and bioaccumulation of the fuel oil in invertebrates. In order to reach these objectives, a bioassay was conducted exposing a population of the polychaete *Arenicola marina* to different dilutions of fuel oil with clean sediment.

## Material and methods

### Toxicity test

Intertidal clean sediment from the Bay of Cádiz (South of Spain) was mixed with fuel oil extracted from the tanker (0.5, 1, 2, 4 and 8% dry weight). The sediment was filtered (0.6 mm) prior to the toxicity to remove inorganic and organic debris and benthic organisms capable of preying *A. marina* (Riba et al. 2003). These sediments were dried and homogenized at room temperature prior to chemical analysis.

The *A. marina* lugworms were sampled in field by hand-digging and immediately transported to laboratory in containers with sea water. Once there, lugworms were placed in aquariums with sieved sediment from the Bay of Cádiz (5 cm thick) and acclimated for 10 days; air was provided and water was replaced three times per day. Water temperature was kept at 18°C and natural photoperiod was selected. The dilutions of fuel oil (0.5, 1, 2, 4 and 8% dry weight) in sediment and the clean sediment without fuel oil (2 kg) were placed in replicates (three) in 11 L tanks and clean sea water was added. Lugworms were put into the tanks (six per tank) which were covered to avoid evaporation. The experiment lasted 21 days. Mortality was daily recorded and after 10 and 21 days of exposure sampling was performed by transferring individuals

to aerated clean sea water without sediment, where they were held for approximately 4 h to empty the sediment of the body. Organisms were then frozen at  $-20^{\circ}\text{C}$ .

#### Chemical analysis

Sediment was digested as described by Loring and Rantala (1992) for trace metal analysis (Ni, V, Cd, Pb, Cr, Co). Measurement was performed by graphite furnace atomic absorption spectrophotometry (Perkin-Elmer 4100 ZL) (USEPA 1984), et al. Results are expressed as milligrams per kilogram of dry sediment. The analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) and showed a recovery greater than 90% of the certified concentration.

Polycyclic aromatic hydrocarbons (fluorene, acenaphthene, naphthalene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[a]fluoranthene, benzo[e]pyrene, benzo[a]pyrene, perylene, dibenzo[ah]anthracene, indene[123-cd]pyrene, benzo[ghi]perilene) were analyzed by using a gas chromatograph equipped with an electron capture detector (GC/MS) (US Environmental Protection Agency 1984). Briefly, dried samples were Soxhlet extracted with *n*-hexane for 18 h, and the extracts were isolated by column chromatography on Florisil–alumino–silica. PAHs were eluted and their fractions were dried in a rotatory evaporator and re-dissolved in iso-octane. Aromatic fractions were analyzed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph coupled with HP 5970 mass spectrometer. Chromatographic resolution was achieved with a  $30\text{ m} \times 0.250\text{ mm}$  DB-5 capillary column, which has a  $0.25\text{ }\mu\text{m}$  film thickness, with helium as carrier gas. Quality control was carried out using NRC-CNRC HS-6 sediment reference material. The analytical procedure showed a recovery greater than 90% of the certified concentration.

The individuals of each treatment were put together and liophilized for the PAHs analysis. Briefly, the samples were Soxhlet extracted with hexane/acetone 1:1 during 24 h; then, the extracts were transferred into tubes and dissolved with hexane until a final volume of 20 ml. Samples of each tube were evaporated to 2 ml. These extracts were isolated by column chromatography on alumino–silica using 20 ml hexane, then 30 ml hexane/methane 9:1

(Fraction I: aliphatic hydrocarbons) and finally 40 ml of hexane/methane 4:1 (Fraction II: PAHs). Flasks with Fraction II were dried in a rotatory evaporator and re-dissolved in hexane (final volume 10 ml). These extracts were evaporated until a final volume of 0.5 ml. Aromatic fractions were analyzed with a gas chromatograph coupled with a mass spectrometer (Finnigan Mat, GCQ tm). Chromatographic resolution was achieved with a  $30\text{ m} \times 0.250\text{ mm}$  DB-5 capillary column, which has a  $0.25\text{ }\mu\text{m}$  film thickness, with helium as carrier gas.

#### Data calculations

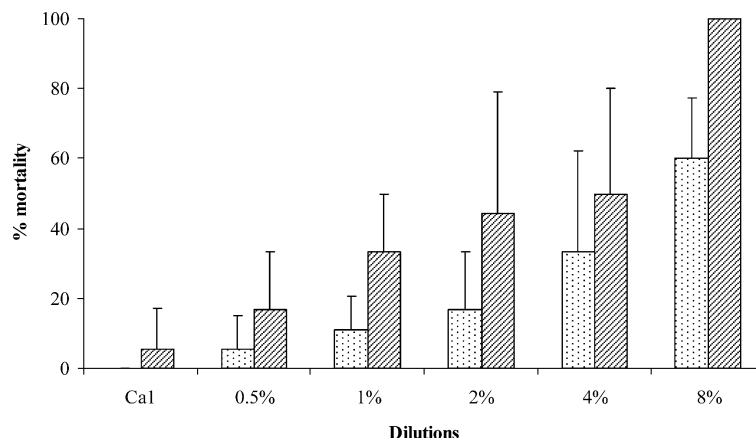
The toxic parameter associated with the fuel oil (LC50) was obtained from the mortality data of *Arenicola marina* measured after 10 and 21 days of exposure to the fuel dilutions (0.5, 1, 2, 4 and 8%). LC50 was defined as the concentration (percentage of fuel oil) that provokes the mortality of 50% of the *Arenicola marina* population exposed. The LC50 was calculated by linear regressions of log toxicant dilution of fuel oil on declining probit values (Probit-Analysis-Program, version 1.5). Sediment Quality Values were calculated basing in the LC50 results.

## Results and discussion

#### Acute toxicity

The mortality of the lugworm *Arenicola marina* in each treatment was recorded after 10 and 21 days of exposure. Figure 1 shows how mortality in control (clean sediment from the Bay of Cádiz) was 0 after 10 days of exposure, while after 21 days 5.6% of the organisms exposed died. However, mean survival in all the replicates of clean sediment from the control (Ca1) was higher than 94% after 10 and 21 of exposure. Death of test organisms was positively related to dose at time of exposure. No survival was detected in the highest dilutions of fuel oil (8%) after 21 days of exposure. For all treatments, the day 21 of exposure shows higher mortality of *Arenicola* than the day 10. Survival results for *Arenicola* show substantial variability among replicates which has been already observed in previous studies (Matthiessen et al. 1998). Other authors obtained differences in tolerance to toxicants, including PAHs, depending on

**Fig. 1** Average and standard deviations of the percentage of mortality of the polychaete *Arenicola marina* after 10 (dotted bars) and 21 (striped bars) days of exposure to each dilution



the polychaete specie (Bach et al. 2005) whereas low percentages of oil contaminated sediment inhibited *Arenicola* feeding almost completely (Grant and Briggs 2002). The mortality of individuals of *A. marina* exposed to dredged material demonstrated a slight correlation with the organic contaminants (PAHs and PCBs) even though these correlations were not significant (Casado-Martínez 2007). Results obtained in this study show a toxicity related to time of exposure, what was previously confirmed by previous studies (Rossi and Neff 1978).

Despite the variability of mortality results in the *Arenicola* bioassays the estimation of LC50 values can be performed. The mortality data were used to calculate two LC50s: LC50(10) to describe toxicity after 10 days of exposure and LC50(21) to describe toxicity after 21 days of exposure. The LC50 value and the concentration of PAHs or metals in the sediment permits calculation of the Sediment Quality Values (SQVs) for each contaminant (Table 1). The LC50(10) value of fuel oil associated with the toxic responses for *Arenicola marina* is 6.4% of fuel oil, which corresponds with a concentration of  $92.42 \text{ mg kg}^{-1}$  of total PAHs ( $[\text{PAH}]_{\text{oil}} \cdot 6.4/100$ ) (SQV<sub>1</sub>). On the other hand the LC50(21) of fuel oil associated with toxicity for this polychaete is 2.4%, which accounts for a concentration of  $34.52 \text{ mg kg}^{-1}$  of total PAHs ( $[\text{PAH}]_{\text{oil}} \cdot 2.4/100$ ) (SQV<sub>2</sub>). Previously, a 10-day exposure study with the amphipod *Corophium volutator* and fuel oil from the tanker produced an LC50 of 1.37% (Morales-Caselles, ICMAN-CSIC, personal observa-

tions) which suggests that *Arenicola* presents lower sensitivity to the fuel than *Corophium*. Previous studies have demonstrated that *Arenicola marina* shows markedly lower acute toxicity to hydrocarbon and other contaminants bound to sediments than *Corophium* (Matthiessen et al. 1998; Grant and Briggs 2002).

The values of total PAHs concentration obtained from the LC50s calculations (SQV<sub>1</sub> and SQV<sub>2</sub>) may be compared with international Sediment Quality Guidelines (SQGs). National Oceanic and Atmospheric Administration (1999) explains the 10th percentile values named the ERL (Effects Range-Low) as the concentrations below which adverse effects rarely occur, whereas the 50th percentiles named ERM (Effects Range-Median) values are representative of concentrations above which effects frequently occur. SQV<sub>1</sub> for total PAHs ( $92.42 \text{ mg kg}^{-1}$ ) is higher than the guidelines ERL and ERM calculated ( $4,022 \text{ } \mu\text{g kg}^{-1}$  and  $44,792 \text{ } \mu\text{g kg}^{-1}$  respectively) while SQV<sub>2</sub> ( $34.52 \text{ mg kg}^{-1}$ ) keeps higher than ERL and lower than ERM. The justification about why the SQVs obtained for *Arenicola marina* are higher than the ERM could be because of the fact that this polychaete species presents lower sensitivity to the PAHs toxicity than other marine organisms, which allows *Arenicola* to survive in an environment highly contaminated by these compounds. On the other hand results of SQVs for metals are lower than the National Oceanic and Atmospheric Administration guidelines, hence metals are probably not a toxicity factor.

**Table 1** Total PAHs and metal concentration measured in the negative control (Ca1) and in the fuel oil

		Fuel	Ca1	ERL	ERM	SQV <sub>1</sub>	SQV <sub>2</sub>
PAHs ( $\mu\text{g kg}^{-1}$ )	Total PAHs	1443	n.d	4022	44792	92424	34517
	Fluorene	99.3	n.d	19	540	—	—
	Acenaphthene	75.3	n.d	16	500	—	—
	Naphthalene	395	n.d	160	2100	—	—
	Phenanthrene	385	n.d	240	1500	—	—
	Anthracene	51.4	n.d	85	1100	—	—
	Fluoranthene	28.5	n.d	600	5100	—	—
	Pyrene	111	n.d	665	2600	—	—
	Benzo[a]anthracene	55.9	n.d	261	1600	—	—
	Chrysene	102	n.d	384	2800	—	—
	Benzo[fluoranthene	16.0	n.d	n.a.	n.a.	—	—
	Benzo[e]pyrene	45.7	n.d	n.a.	n.a.	—	—
	Benzo[a]pyrene	29.7	n.d	430	1600	—	—
	Perilene	11.4	n.d	n.a.	n.a.	—	—
	Dibenzo[ah]anthracene	5.70	n.d	63	260	—	—
	Indene[123-cd]pyrene	5.23	n.d	n.a.	n.a.	—	—
	Benzo[ghi]perilene	17.1	n.d	n.a.	n.a.	—	—
Metals ( $\text{mg kg}^{-1}$ )	Ni	55	14.1	20.9	51.6	8.3	7.5
	V	170	80.0	n.a.	n.a.	42.7	41.1
	Cd	n.d	n.d	1.2	9.6	n.d	n.d
	Pb	n.d	23.0	46.7	218	10.8	11.2
	Cr	0.31	31.0	81	370	14.6	15.1
	Co	n.d	3.40	n.a.	n.a.	1.6	1.7

Sediment quality values for PAHs are obtained from the LC50 and calculated for the *Arenicola marina* used in the sediment toxicity test. Sediment Quality Guidelines were derived using previous studies data (ERL= Effects Range-Low and ERM= Effects Range-Median, National Oceanic and Atmospheric Administration (1999) (n.d, not detected; n.a., not analyzed).

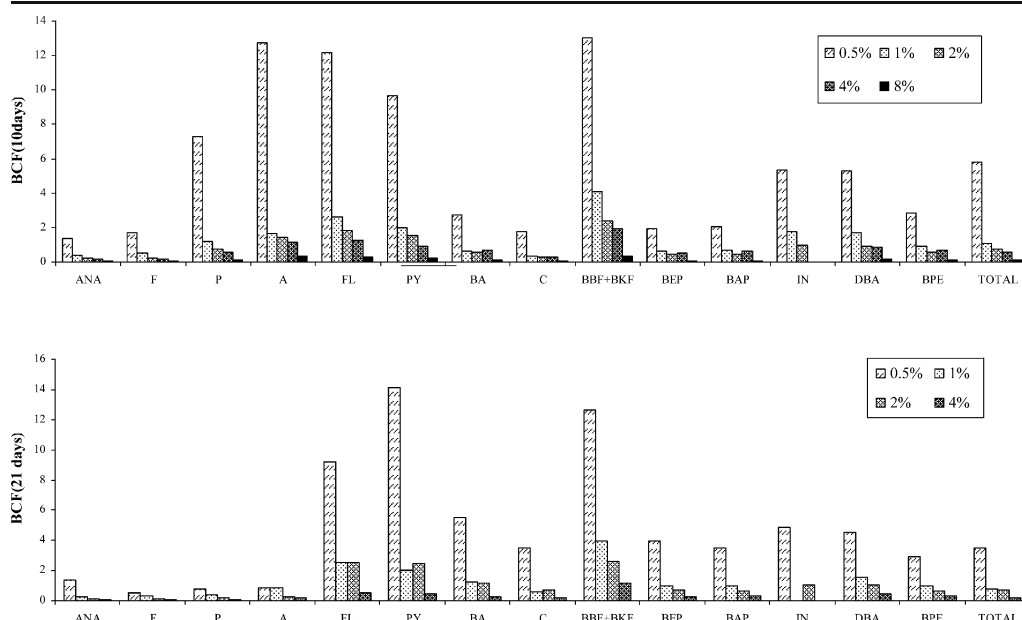
### Bioaccumulation

The polychaetes ingest sediment and thus are exposed to PAHs in solution in the interstitial water and those adsorbed to sediment particles (Neff 2002).

Organisms were sampled the day 10 and 21 of the fuel oil exposure experiment in order to analyze the content of PAHs in their bodies. A biota/sediment bioaccumulation factor (BCF) was defined to interpret the results obtained. This factor accounts for the concentration of PAHs in the organisms (Co) related to the concentration of that contaminant in the sediment (Cs) ( $\text{BCF} = \text{Co}/\text{Cs}$ ). BCF results are shown in Fig. 2. In general, bioaccumulation decreases when the percentage of fuel in the sediment sample increases. This behaviour could be due to the fact that toxicity increases with the content of fuel. Casts were not found in those tanks with higher concentrations of hydrocarbons, so probably feeding was inhibited by the presence of contaminants in the sediment; this fact could lead to lower levels of bioaccumulation in those treatments with

the highest amount of fuel oil. This trend has been shown in previous studies on other invertebrates (Landrum et al. 2003) where, in general, the uptake coefficient declined with increasing PAHs concentration, especially with pyrene. Also, lower accumulation factors were found to correspond to treatments for which significant mortality was observed (Rust et al. 2004a).

The BCF calculated for the day 10 of exposure is higher for phenanthrene, anthracene, fluoranthene, pyrene and benzo[fluoranthenes (Fig. 2); after 21 days of exposure the highest levels of bioaccumulation were for fluoranthene, pyrene and benzo[fluoranthenes but not for the lower molecular weight compounds phenanthrene and anthracene. The decreased of the BCF for phenanthrene and anthracene could be due to the fact that after 21 days the organisms have been able to metabolize the PAHs with lower molecular weight. On the other hand, during long-term contact between PAHs and sediment particles, PAHs become tightly bound to organic phases in the sediment, reducing their bioavailability (Neff 2002).



**Fig. 2** Bioaccumulation factors (BCF) calculated for *Arenicola marina* after 10 and 21 days of exposure to the dilutions of fuel oil (0.5, 1, 2, 4 and 8%). ANA Acenaphthene, F fluorene, P phenanthrene, A anthracene, FL fluoranthene, PY pyrene, BA benzoanthracene, C chrysene, BBF + BKF benzo(b)fluoran-

thene and Benzo(k)fluoranthene, BEP benzo[e]pyrene, BAP benzo[a]pyrene, IN indene[123-cd]pyrene, DBA dibenzo[ah]anthracene, BPE benzo[ghi]perilene, TOTAL sum of individual PAHs

Levels of BCF confirm that those PAHs that present logKow values of 5–6 show the highest accumulation potential as reported in previous studies (Kaag et al. 1997; kaag et al. 1998; Rust et al. 2004a).

Fluoranthene presents high bioaccumulation potential relative to smaller or larger PAHs and it is known to be highly toxic to benthic invertebrates (Selck et al. 2003; Landrum 1989; Swartz et al. 1990). This compound may also possess genotoxic (mutagenic and carcinogenic) properties, though these effects are not associated directly with the parent compound, but arise largely as a result of biotransformation processes that lead to the formation of reactive intermediates (Rastetter et al. 1982; Babson et al. 1986; Bach et al. 2005). Pyrene presents a high bioaccumulation factor and associates strongly to sediment particles (Landrum 1989). Results of BCF for pyrene increase from day 10 to day 21, in contrast with other authors that found that the fraction of unmetabolized pyrene in tissues of *A. marina* was unaffected by the duration of exposure (Christensen et al. 2002). Benzo(b)fluoranthene and benzo(k)fluoranthene present similar values of BCF for the day 10

and 21 of exposure, which suggests that these PAHs with high molecular weight were initially bound to the organism tissues and were not metabolized probably due to their low solubility. On the other hand this could be as a result of the fact that threshold effect has been achieved and the availability of the high molecular weight compounds decreases as a consequence of tight organic bonding in the sediment.

## Conclusions

In the present study the sensitivity of the polychaete *Arenicola marina* to the fuel oil from a tanker (*Prestige* 2002) has been tested, and in spite of the variability in mortality results, it showed a clear dose-related mortality but more endurance than other organisms. Bioaccumulation was mainly produced for fluoranthene, pyrene, benzo(b)fluoranthene and benzo(k)fluoranthene whereas phenanthrene and anthracene were initially accumulated and then probably metabolized.

Although PAHs do not biomagnify through trophic levels (Neff 2002), *A. marina*, which is often used as a bait, is able to live in PAH-contaminated environments and accumulate PAHs. Although *A. marina* is less sensitive than other species, it is likely to be available even in at polluted sites, for studies of PAH bioaccumulation. Attending to this, we propose that *Arenicola marina* should be used in the assessment of oil impacts associated with spills included in a set of bioassays, in order to determine acute and sublethal toxicity responses; in addition further research towards including biomarkers in this species it is recommended.

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## Comparing sediment quality in Spanish littoral areas affected by acute (*Prestige*, 2002) and chronic (Bay of Algeciras) oil spills

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*Littoral sediments affected by low or moderated but continuous oil spills are more polluted than those affected by accidental oil spills such as the Prestige.*

### Abstract

The quality of sediments collected from two areas of the Spanish coast affected by different sources of contaminants has been compared in this study. The areas studied are the coast of Galicia affected by the oil spill from the tanker *Prestige* (November 2002) and the Gulf of Cádiz which suffers continuous inputs of contaminants from industries located in the area and from oil spills. Contamination by several chemicals (metals, PCBs and PAHs) that bind to sediments was analyzed, and two toxicity tests (Microtox<sup>®</sup> and amphipod 10-day bioassay) were conducted. PAHs were identified as the compounds responsible for the toxic effects. Results show differences between an acute impact related to the sinking of the tanker *Prestige* and the chronic impact associated with continuous oil spills associated with the maritime and industrial activities in the Bay of Algeciras, this being the most polluted part of the two coastal areas studied in this work.

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**Keywords:** Amphipods; Microtox<sup>®</sup>; PAHs; Toxicity; Contamination

### 1. Introduction

Sediments are an important part of the ecosystem and play a key role in the distribution of contaminants in the aquatic environment; the study of the quality of sediments provides information about the ecosystem health. Human activities in coastal areas usually involve an input of contaminants to the natural environment that becomes evident in the decreased quality of coastal sediments. Many authors agree that sediment quality is best determined by integrating the information

obtained from measures of chemicals concentration and from specific tests to determine sediment toxicity (DelValls and Conradi, 2000; Chapman et al., 2002). The biological effects can be established based on laboratory tests that determine toxic responses. Sediment bioassays are usually relatively simple tests that evaluate the responses of the tested organism to contaminated sediments under controlled conditions (Riba et al., 2004a).

In the present study, we have selected two different tests in order to determine sediment toxicity: the Microtox<sup>®</sup> test and an amphipod acute bioassay. Use of the commercial bioassay Microtox<sup>®</sup> has increased in recent years since it detects the “hot spots” of field contamination in the screening procedure (Mowat and Bundy, 2001; Stronkhorst et al., 2003; Van Beelen, 2003); Microtox<sup>®</sup> has also been used before to assess the impact of oil spills and oil contaminated

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sediments (Brohon et al., 2001; Kenneth et al., 2003; Pelletier et al., 2004). The other acute bioassay was carried out with the amphipod *Corophium volutator* which is an important test organism for the ecotoxicological quality assessment of marine and estuarine sediment samples (Peters and Ahlf, 2005).

The bioassay with *C. volutator* is integrated in test batteries for dredged material management (Peters et al., 2002; Stronkhorst et al., 2003) and it is required for compliance with certain International Standardization Organisation quality standards (ISO, 2003). This kind of bioassay has been used in previous studies for the assessment of spills (Grant and Briggs, 2002; Briggs et al., 2003).

In this study a comparison is made between the quality of sediments sampled on two lengths of Spanish coast affected by different sources of contaminants. In the coast of Galicia, the study addresses the acute impact provoked by the oil spill resulting from the break-up and sinking of the tanker *Pres-tige* (November 2002), whereas in the Gulf of Cádiz the sediments studied have suffered a chronic impact lasting several decades, caused by the input of oil and other contaminants from the various industries located in the area and from accidental spills and deliberate discharges from commercial shipping activities.

The main objectives of this study are: (1) to characterise the contamination by PAHs in the selected areas of study on the Galician Coast and in the Gulf of Cádiz; (2) to establish the sediment toxicity caused by the presence of contaminants in the sediment samples; (3) to compare the sediment quality of the various areas studied by linking contamination and ecotoxicological data.

## 2. Materials and methods

### 2.1. Approach

Fig. 1 shows the seven sediment sampling stations located in the National park of the Atlantic Islands that were selected in the area of Galicia, three stations in the island of Ons (D07, D09 and D18) and four stations in the Cíes archipelago (D60, D66, D79 and FIG). In the Gulf of Cádiz seven stations were selected in the area of the Bay of Algeciras: three stations in the mouth of the river Guadarranque (GR1, which is near an oil-fired electricity generating plant, GR3' and GR4, both near chemical processing plants), one station in the mouth of the river Palmones (P4) and three stations in the Bay (AL1 and AL2, both located in the port and near the city of Algeciras, and AL5, near a chemical plant). All these sediments have suffered repeated impacts by moderate or small oil spills caused by maritime traffic and bunkering activities in the area during recent decades. Clean sediment from the Bay of Cádiz was used as the negative control (Ca1). An artificial sample (TM) was made by mixing a toxic mud from an accidental mining spill in Spain (Aznalcóllar, April 1998) with the same clean sediment and used as positive toxicity control (Riba et al., 2003).

Sediments were collected with a 0.025 m<sup>2</sup> Van Veen grab and transferred to the cooler. When sufficient sediment had been collected from a particular station, the cooler was transported to the laboratory. The contents of the cooler were homogenized with a Teflon spoon until no colour or textural differences could be detected. The samples were subsampled for physical characterization and chemical quantification. After that, sediment samples were maintained in the cooler at 4 °C in the dark until they were used for sediment toxicity testing, but no longer than 2 weeks.

### 2.2. Chemical analysis

Sediment aliquots for chemical analysis were dried at room temperature and then gently homogenized. Total organic carbon (TOC) concentration and sediment grain size (fines: % of dry sediment < 63 µm) were studied in order to determine the geochemical matrix characteristics. Organic carbon content was determined using the method of Gaudette et al. (1974) with the El Rayis (1985) modification. For sediment grain size, an aliquot of wet sediment was analyzed using a Frisch laser particle sizer (model Analysette 22) following the method reported by DelValls and Chapman (1998).

For trace metal analysis, the sediments were digested as described by Loring and Rantala (1992). Zn and Cu concentrations in the extracts were determined using a Perkin–Elmer 2100 flame atomic absorption spectrophotometer. The other trace metals were measured by graphite furnace atomic absorption spectrophotometry (Perkin–Elmer 4100 ZL). Concentrations of Hg were determined using a Perkin–Elmer MHS-FIAS coupled with a Perkin–Elmer 4100 ZL spectrophotometer. Results are expressed as mg kg<sup>-1</sup> dry sediment. The analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) and showed agreement with the certified values of more than 90%.

The analyses of PAHs and PCBs were carried out according to USEPA SW-846 Method 827C78082. Briefly, following recommendations by Riba et al. (2002), dried samples were Soxhlet extracted with *n*-hexane for 18 h, and the extracts were isolated by column chromatography on Florisil–alumino-silica. PCBs and PAHs were eluted and their fractions were dried in a rotating evaporator and re-dissolved in isooctane. Aromatic fractions were analyzed on a Hewlett–Packard (HP) 5890 Series II gas chromatograph coupled with an HP 5970 mass spectrometer. Chromatographic resolution was achieved with a 30 m × 0.250 mm DB-5 capillary column, which has a 0.25 µm film thickness, with helium as carrier gas. The 16 priority PAHs considered by the US Environmental Protection Agency were analyzed by GC–MS using selected ion monitoring (SIM). Quality control was carried out using NRC-CNRC HS-6 sediment reference material. Analysis of PCBs as AROCLOR 1242 and AROCLOR 1260 was performed using the same instrument with an electron capture detector (GC/ECD) and a 30 m × 0.25 mm MDN-5S capillary column. Quantification was performed by the external standard technique by comparison of peak areas in the sample with those obtained by injecting a standard mixture of AROCLOR 1242 and 1260. Quality control was carried out with NRC-CNRC HS-1 sediment reference material. For both set of organic chemicals, PAHs and AROCLOR, the analytical procedure showed agreement with the certified values of more than 90%.

### 2.3. Microtox® bioassay

The commercial Microtox® test is a bioassay that uses the bioluminescence of the bacteria *Vibrio fischeri* as an indicator of the quality of the sample (liquid or solid phase) exposed; the bioluminescence of the bacteria is related to its metabolism therefore, a diminution of the sediment quality will be reflected in the decrease of the quantity of light emitted. In the Microtox® toxicity test, the measures of the IC50 (which is the concentration of dry sediment that provokes a 50% inhibition of the light emitted by the bacteria) provide the information about the sediment toxicity. The bioassay of bioluminescence inhibition with the bacteria *V. fischeri* was conducted on solid phase with the commercial Microtox® apparatus (model 500) by following the protocols for the solid phase test (SPT) according to the standard operating procedure (AZUR Environmental, 1998). Briefly, 7 g (±0.01 g) of sediment were tested as suspensions prepared with 35 mL of commercial Microtox® Solid Phase Test Diluent and diluted to a series of nine concentrations in the cuvettes. The reconstituted bacteria were added to the dilutions which were incubated for a period of 20 min at 15 °C in a waterbath. Next the dilutions were filtered through the filter columns and 500 mL of content of each cuvette of the bath were transferred to its corresponding cuvette in the apparatus and bioluminescence was measured in the “read well”. The modification of the basic solid phase test (BSPT) reported by Campisi et al. (2005) was carried out and an average value of the IC50 from using both protocols was obtained for each sample.

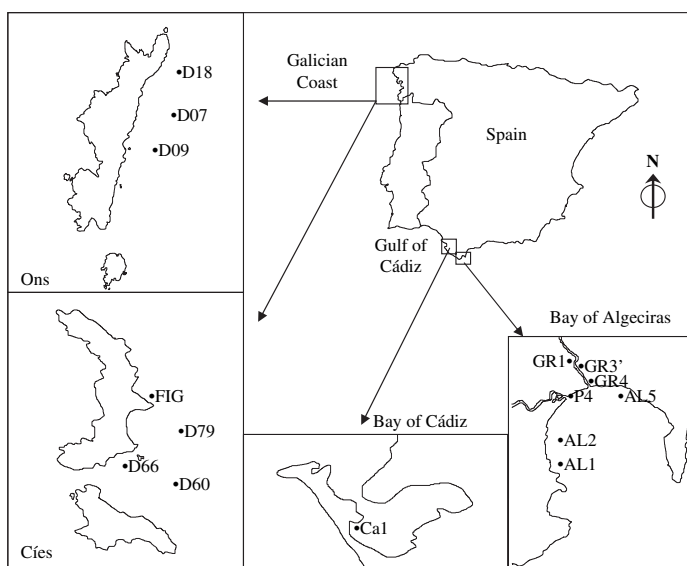


Fig. 1. Map of the coastal area of Galicia, the Bay of Algeciras and the Bay of Cádiz showing the general areas sampled and locations of the sampling stations. D(#) refers to the stations located in Galicia and AL(#), P(#), and GR(#) to those in the Bay of Algeciras. Ca1 was the station selected as negative control in the Bay of Cádiz.

## 2.4. Amphipod bioassay

Individuals of *C. volutator* were obtained from the field in a clean area located in the coast of Galicia (Morales-Caselles, 2005) by sieving mud through a 1 mm mesh; when the organisms were isolated they were placed in 11 L capacity aquariums with clean seawater and sieved sediment (collected in the same area as the organisms) and were maintained in the laboratory under controlled conditions for acclimation until the start of the test. Aeration was provided and natural photoperiod was selected. During acclimation (1 month) the organisms were fed twice a week with food for invertebrates ("Marine Invertebrate Diet" which is a mixture of amino acids and organic particles) and water was replaced.

The toxicity test was conducted in replicate (5) by exposing individuals of the amphipods *C. volutator* to bulk sediment using the percentage of survival after 10 days of exposure as the end point (ASTM, 1993). Mortality is measured after the time of exposure and the results obtained have been correlated positively with changes in benthic communities (Long et al., 2001).

Approximately, 250 g of sieved (1 mm) sediment was placed in 2 L glass containers and then about 750 mL of clean seawater were added. Aeration was provided after the sediment had settled down. The individuals of *Corophium* were sieved and separated from sediment of the acclimating tanks and were placed in each replicate container (20 individuals per container). The containers were covered in order to avoid water evaporation (a hole was made in the lid to provide aeration), and maintained at 19 °C during the 10 days of exposure. After that time, the contents from the different stations with the various replicates were sieved and the organisms' survival rate was recorded.

## 2.5. Statistical analysis

ANOVA was performed in order to determine significant differences ( $p < 0.05$ ) in amphipod survival among the toxicity results obtained for the control site and the other sampling sites. Also, contamination and toxicity data were linked by factor analysis, using principal components analysis (PCA) as the extraction procedure; this is a multivariate statistical technique for exploring variable distributions (Riba et al., 2003). The original data set

used in the analysis included two acute toxicity responses (amphipod survival and the IC50 measured in the Microtox® bioassay), the sediment concentration of different contaminants (PAHs, PCBs, Cd, Cu, Ni, Co, V, Pb, Zn, Hg), and the geochemical matrix characteristics (including total organic carbon and grain size distributions). The objective of PCA is to derive a reduced number of new variables as linear combinations of the original variables. This provides a description of the structure of the data with the minimum loss of information.

## 3. Results

### 3.1. Chemical analysis

Summarized results of chemical analyses in the sediments used for both bioassays are shown in Table 1. In general, results do not show a prevailing tendency in the concentration of metals among sediments from the different areas, although the toxic mud presents the highest concentration of metals. On the other hand, organic contaminants (PAHs and PCBs) seem to be at higher levels in sediments collected in the Bay of Algeciras than in the area of Galicia. The negative control (Ca1) shows the lowest levels of metals, and no organic contamination was found.

### 3.2. Microtox® bioassay

The highest inhibition of bioluminescence, which corresponds to an IC50  $< 600 \text{ mg L}^{-1}$  dry weight, is shown in the samples collected at the Bay of Algeciras stations AL2 ( $69 \text{ mg L}^{-1}$ ), AL1 ( $208 \text{ mg L}^{-1}$ ), GR3' ( $235 \text{ mg L}^{-1}$ ), GR4 ( $249 \text{ mg L}^{-1}$ ) and GR1 ( $522 \text{ mg L}^{-1}$ ), and in the sediments from the coast of Galicia D60 ( $358 \text{ mg L}^{-1}$ ), D79 ( $364 \text{ mg L}^{-1}$ ), D18 ( $390 \text{ mg L}^{-1}$ ) and D66 ( $486 \text{ mg L}^{-1}$ ).

Table 1

Average values of total organic carbon (%dry weight), fines (% of dry sediment < 63  $\mu\text{m}$ ) and the concentration of contaminants (metals ( $\text{mg kg}^{-1}$  dry weight); PAHs and PCBs ( $\mu\text{g kg}^{-1}$  dry weight)) in sediment samples (negative control: Ca1; positive control: TM; Algeciras Bay: GR1, GR3', GR4, P4, AL1, AL2, AL5; Galicia: D07, D09, D18, D60, D66, D79 and FIG.) Not detected is expressed by n.d.

	TOC	Fines	Zn	Cd	Pb	Cu	Ni	Co	V	Hg	PAH	PCBs
Ca1	1.07	1.04	21.3	0.92	2.31	6.98	0.06	3.40	80.0	n.d.	n.d.	n.d.
GR1	3.12	75.1	44.8	0.61	9.10	12.6	6.01	n.d.	6.69	n.d.	546	0.86
GR3'	2.15	89.2	138	0.17	22.0	5.01	74.7	12.8	26.1	1.04	2961	22.0
GR4	3.19	39.5	35.3	0.10	6.21	3.67	13.1	5.59	n.d.	0.25	802	1.75
P4	2.09	34	50.4	0.62	5.64	11.3	24.7	1.11	85.0	0.11	21.4	4.64
AL1	2.35	92.3	137	0.16	32.0	30.5	50.9	n.d.	60.8	1.11	1383	0.46
AL2	3.22	90.8	54.0	0.11	9.81	7.59	15.1	1.69	4.82	0.81	1376	0.65
AL5	1.22	3.2	23.0	0.14	7.12	10.8	52.6	5.09	2.19	0.26	1218	0.33
D07	3.79	45.6	85.3	n.d.	23.1	251	1.04	n.d.	81.2	0.08	465	n.d.
D09	4.61	59.9	107	n.d.	28.3	160	11.7	n.d.	116	0.07	240	n.d.
D18	2.42	12.9	55.5	n.d.	14.2	20.8	3.44	2.00	54.0	0.04	480	n.d.
D60	3.56	60.9	101	n.d.	31.0	70.9	16.2	n.d.	125	0.12	702	n.d.
D66	0.37	11.3	14.0	n.d.	4.10	16.2	4.60	0.30	n.d.	0.06	384	n.d.
D79	3.58	70.2	114	n.d.	29.1	150	4.44	n.d.	13.7	0.09	273	n.d.
FIG	2.10	2.12	76.2	n.d.	26	18.5	11.8	0.50	n.d.	0.04	390	n.d.
TM	1.00	10.1	2181	5.40	789	210	8.5	n.d.	n.d.	5.61	n.d.	n.d.

The positive control of toxicity TM shows a 50% inhibition of bioluminescence at very low concentration ( $142 \text{ mg L}^{-1}$ ). The sediment obtained from the Bay of Cádiz and used as the clean reference (station Ca1), showed the highest value of IC<sub>50</sub> ( $6013 \text{ mg L}^{-1}$ ) and confirms its validity as the negative toxicity control. Microtox<sup>®</sup> results are shown in Fig. 2.

### 3.3. Amphipod bioassay

Mean mortality results after the 10 days amphipod toxicity test are shown in Fig. 3. The highest mortality measurements (100%) were associated with sediments found in the Bay of Algeciras at station GR3' and with the positive control of toxicity TM, while lowest mortality (3.3%) was associated with sediments from the reference station Ca1. The mortality

measured in the sediments from GR3' (100%), TM (100%), GR4 (75%), D18 (45%), D66 (60%), P4 (33.3%) and AL5 (35%) was significantly different  $*p < 0.05$  from the negative control Ca1. The other stations did not present significant differences in the mortality results compared to the control station (Ca1).

### 3.4. Statistical analysis

To link the set of data obtained, the original variables from chemical concentration and toxicity responses were analyzed by factor analysis, using principal components analysis (PCA) as the extraction procedure; this is a multivariate statistical technique (MAA) for exploring distributions of the variables (chemical concentration,  $n = 14$ ; toxicity data,  $n = 2$ ).

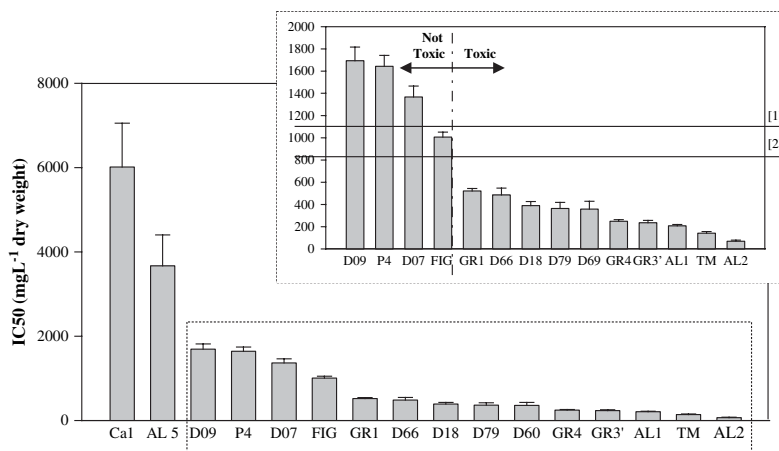


Fig. 2. IC<sub>50</sub> results obtained from the application of the Microtox<sup>®</sup> test to sediment samples from the various stations. A zoom is provided for IC<sub>50</sub> <  $1800 \text{ mg L}^{-1}$  (dry weight). Lines displayed show the limits below which the sediment sample is considered toxic by the Canadian Standards ( $1000 \text{ mg L}^{-1}$  dry weight) and by the proposed Spanish Standards (Casado-Martínez et al., in press),  $750 \text{ mg L}^{-1}$  (dry weight).

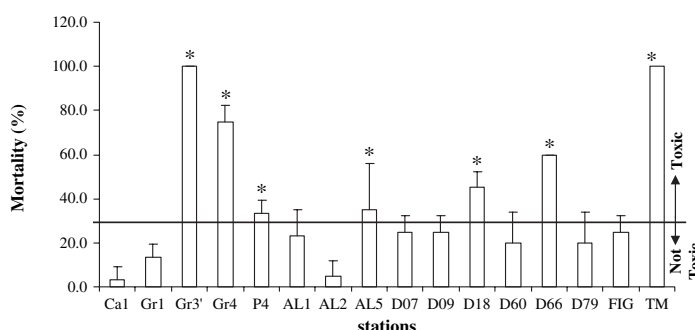


Fig. 3. Mortality results after 10 days of exposing *C. volutator* to the sediment samples. Asterisks indicate significant differences between the amphipod mortality rate in the treatments and the negative control (\* $p < 0.05$ ). The line displayed shows the limits below which the sediment samples are considered toxic by the proposed Spanish Standards (DelValls et al., 2004; Casado-Martínez et al., in press). Those samples where the mortality rate of the amphipods is 20% higher than the mortality recorded in the negative control (Ca1) and show significantly different (\* $p < 0.05$ ) results compared to those obtained in Ca1, are considered as toxic.

The factor analysis was performed on the correlation matrix; the variables were autoscaled (standardized) so as to be treated with equal importance (Riba et al., 2004a). The application of MAA to the original 14 variables indicates that they can be grouped in three new factors. These factors explain 78.3% of the total variance in the original data set. In the present study, we decided to interpret a group of variables as those associated with a particular component where the loading was 0.30 or higher (Table 2). This approximates to Comreys' cut-off of 0.55 (Comreys, 1973) for a good association between an original variable and a factor, and also takes into account discontinuities in the magnitudes of loadings approximating the original variables.

The first principal factor, #1 is predominant and accounts for 35.0% of the variance; it shows the toxicity to the bacteria and the amphipods associated with the presence of trace metals in sediments (Zn, Cd, Pb, Cu and Hg). The second

factor, #2 accounts for 28.8% of the variance; it explains the amphipods and bacteria toxicity associated with the chemical concentrations of the metals Co and Ni, the organic contaminants PAHs and PCBs. The third factor, #3 accounts for 14.5% of the variance; it shows the relationship between the grain size and the total organic carbon in the sediments with the presence of Cu and V, but toxicity does not contribute to this factor.

The influence of the three factors at the 16 stations is reflected by the Factor score at these stations and is shown in Fig. 4. The definition of Factor 1, with positive loading, is the acute toxicity of the organisms to metals bound to sediment, it is mainly prevalent in the positive control TM (3.65) followed by GR3' (0.02) and AL2 (0.01) with low prevalence. Factor 2 is defined as the lethal toxicity of the amphipods related to the concentration of metals Co and Ni and to the organic compounds, mainly PAHs, bound to sediments; this factor shows significant prevalence in the stations from the Bay of Algeciras: GR4 (0.49), AL1 (0.49), AL2 (0.47), AL5 (0.61) and mainly in GR3' (3.28). The definition of Factor 3, with negative loading, does not include information about the toxicity of the contaminants, but indicates the association of geochemical features of the sediment, described by the relationship between total organic carbon and grain size.

#### 4. Discussion

The chemical data obtained in the analyses show how the levels of PAHs are, in general, higher at the stations in the Bay of Algeciras than in the stations selected on the coast of Galicia. These chemical data can be compared to international sediment quality guidelines (SQGs) that specify the levels of chemical contaminants associated with biological effects (DelValls et al., 2004). In this regard, the samples collected at GR3', AL1, AL2 and AL5 exceed the SQGs for PAHs defined by Dutch agencies (Tweede Kamer, vergaderjaar, 1994–1995); this implies that the sediments from these locations could be considered slightly or moderately polluted

Table 2

Sorted rotated factor loadings (pattern) of 14 variables for the three principal factors resulting from the multivariate analysis of results obtained from the chemical analysis and the acute toxicity tests (Microtox® and *C. volutator* bioassay)

%Variance	Factor 1	Factor 2	Factor 3
	35.01	28.76	14.54
TOC	—	—	0.86
Fines	—	0.40	0.84
Zn	0.98	—	—
Cd	0.93	—	—
Pb	0.98	—	—
Cu	0.51	—	0.42
Ni	—	0.86	—
Co	—	0.88	—
V	—	—	0.41
Hg	0.98	—	—
PAH	—	0.94	—
PCBs	—	0.88	—
Microtox®	0.43	0.30	—
<i>Corophium</i>	0.59	0.52	—

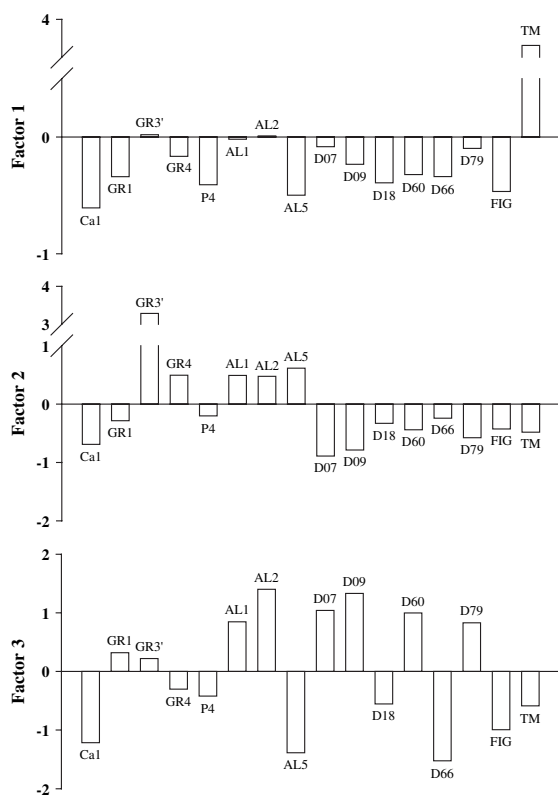


Fig. 4. Estimated factor scores for the three factors in each of the 16 cases. The factor scores quantify the prevalence of each factor for every station and is used to establish the definition of each factor.

according to the Dutch SQGs for PAHs. Following the recommendations described by MacDonald et al. (1996), the sediments from GR4 and D60 would also be considered as slightly polluted by this contaminant and adverse effects could be frequent. There are some stations in the Galician islands (D07, D09, D18, D60, D79 and FIG) where sediment exceeds the SQGs defined for Cu by international agencies and previous studies (CEDEX, 1994; Tweede Kamer, vergaderjaar, 1994–1995; MacDonald et al., 1996; NOAA, 1999). Although contamination by copper was observed in the uppermost layer in the *Prestige* shipwreck area of the Northeast Atlantic Ocean (Prego and Cobelo-García, 2004; Cobelo-García et al., 2004), this Cu contamination should not be related to the shipwreck, because levels of Cu in the fuel oil carried by the *Prestige* were relatively low ( $3.39 \text{ mg kg}^{-1}$ ) and previous studies have shown that there are other sources of this metal in the area (Carballeira et al., 1997). Also, there are some stations in the Bay of Algeciras (GR3', AL1 and AL5) where sediment exceeds some international SQGs (CEDEX, 1994; Tweede Kamer, vergaderjaar, 1994–1995; MacDonald et al., 1996; NOAA, 1999) defined for Ni and for Hg (GR3', GR4, AL1, AL2 and AL5). The positive control TM exceeds almost all

the SQGs defined by international agencies and other authors (CEDEX, 1994; Tweede Kamer, vergaderjaar, 1994–1995; MacDonald et al., 1996; NOAA, 1999) for the metals Zn, Cd, Pb, Hg and Cu.

In Fig. 2 the Microtox® results are shown. The lines represent the values below which sediment toxicity is assumed by different international agencies (Casado-Martínez et al., in press). The Canadian Standards (Environment Canada, 2002) considers the limit to be  $1000 \text{ mg L}^{-1}$  (dry weight) while in the Spanish Standards (DelValls et al., 2004; Casado-Martínez et al., in press) this limit is associated with a concentration of  $750 \text{ mg L}^{-1}$  (dry weight). In this case both guidelines agree and the sediments from GR1, D66, D18, D79, D69, GR4, GR3', AL1, TM and AL2 would all be considered as toxic.

The line displayed in the graph for the *Corophium* mortality (Fig. 3) shows the limit above which sediments are considered toxic by the US Environmental Protection Agency (USEPA, 1994) and the Spanish Standards (DelValls et al., 2004; Casado-Martínez et al., in press). These agencies establish that a sediment sample can be considered toxic when the mortality rate recorded from the treatment is 20% higher than the mortality measured in the negative control sediment; it also shows significantly different ( $*p < 0.05$ ) mortality results compared with those obtained in the negative control. In this case all the samples that are significantly different ( $*p < 0.05$ ) from Ca1 would be considered toxic by these agencies because they also have a mortality rate higher than 20% compared to the control sediment (10%).

From the MMA performed to link together the chemical and ecotoxicological data, we have obtained three factors that account for all the variables and have a different influence for each sampling site. Factor 1 accounts for the toxicity responses of the two bioassays due to the metals that bind to sediments. This factor is seen with most prevalence in the positive control TM which presents the highest levels of metals in the study, a low IC50 and a 100% mortality of *Corophium* after 10 days of exposure.

Regarding the toxicity due to the presence of organic contaminants in the sediments, Factor 2 is mainly prevalent at the stations in the Bay of Algeciras: GR3', GR4, AL1, AL2 and AL5. This factor also includes toxicity due to the metals Ni and Co and the toxicity is determined by the mortality of the amphipods and the low IC50 measured with the bacteria bioassay. The content of PCBs in the sediments studied does not exceed the SQGs and the highest concentration of PCBs is found in sediments from GR3' whereas in the Galician sediments, it was not found at all. Thus, the PAHs can be considered as the main organic contaminant producing the toxicity measured in the study. The metals Ni and Co have been previously reported associated with PAHs in the oil spills occurring in the area of Algeciras (CSIC, 2005) caused both by the industrial plants located in the area and by maritime activities, and in other areas affected by oil spills (Massoud et al., 1998). However, both of these metals (Co and Ni) could originate from any of the various local activities, not only accidental oil spills. The toxicity results from Microtox® have a low loading in this factor, maybe because this is not the best test for

organic compounds, due to the insoluble nature of most of the oil compounds (Simon et al., 2004). However, it is important to highlight that the Microtox<sup>®</sup> is a screening bioassay and it is suggested that the biotests alone may not be representative in certain cases of the full impact of a given pollutant on an ecosystem (Brohon et al., 2001). On the other hand, previous studies have shown that the toxicity of sediments to *Corophium* is closely correlated with their hydrocarbon content (Grant and Briggs, 2002).

In general, results obtained in the MAA suggest that the sediments from the Bay of Algeciras and the Galician islands are contaminated with PAHs and that toxic responses due to these compounds occur in both places. However, toxicity is mainly seen in sediments from the Bay of Algeciras, and toxic sediments show a higher frequency in the stations located in this area (GR1, GR3', GR4, P4, AL1, AL2 and AL5) than in those sampled on the coast of Galicia (D07, D09, D18, D60, D66, D79 and FIG). This could be associated either with the higher quantity of PAHs or with the presence of a more complex mixture of contaminants that has not been analyzed in this study, in sediments from the Bay of Algeciras rather than in those from Galicia. Also, based on the results obtained in this study, the concentration of PAHs bound to sediments in the Bay of Algeciras could be more bioavailable for the organisms studied and would produce more toxicity than that found in Galicia. However, this greater bioavailability of PAHs bound to sediments in the Bay of Algeciras is only a hypothesis that needs to be taken into account in later work.

Sediment quality guidelines (benchmarks) for PAHs can be determined with the information obtained in this study by means of MAA using the Factor scores. The highest concentrations of PAHs measured at those stations where the value of the Factor 2 score is 0 or below 0, define the concentration of PAHs "not associated with the toxic effect" measured in the study. It is determined at station D60 with a value of  $702 \mu\text{g kg}^{-1}$  (dry weight) (V1). The lowest concentration of PAHs measured at the station where the value of Factor 2 score is positive defines the concentration of PAHs "associated with the toxic effect measured in this study". It is determined at station GR4 with a value of  $802 \mu\text{g kg}^{-1}$  (dry weight) (V2).

In general, our results have shown that the highest pollution measured in the Gulf of Cádiz was determined in sediments sampled from the Bay of Algeciras and especially at station GR3', related to chronic contamination, while moderate contamination and low toxicity was determined on the coast of Galicia in the Cíes archipelago. Previous studies (Riba et al., 2004b) agree that moderate but chronic inputs of contaminants can produce more pollution in coastal sediments than higher but acute environmental impacts.

## 5. Conclusions

From the results obtained in the various analyses performed in this study, we can conclude that PAHs are the main contaminant at the sites studied on the coast of Galicia and in the Bay of Algeciras, while there is no such contamination in the sediments from the station located in the Bay of Cádiz; the

concentrations of PAHs in sediments from the Bay of Algeciras are, in general, higher than in sediments from the coast of Galicia. Toxicity is also higher in the Bay of Algeciras than in the Galician islands, but no toxicity was detected in the sediment from the Bay of Cádiz station. Finally, it has been shown that sediments from the Bay of Algeciras are chronically polluted by PAHs (V1:  $702 \mu\text{g kg}^{-1}$ ; V2:  $802 \mu\text{g kg}^{-1}$ ) while those of the coast of Galicia can only be considered as moderately or not polluted. In the Bay of Cádiz no environmental degradation was measured.

To sum up, with the present study we have shown that sediments found in the Bay of Algeciras, affected by chronic oil spills, are more environmentally degraded (polluted) than those found in the coast of Galicia, which was mainly affected by the *Prestige* accidental oil spill.

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# **Sediment contamination, bioavailability and toxicity of sediments affected by an acute oil spill. Four years after the sinking of the tanker prestige (2002).**

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## **Abstract**

Sediment contamination and three bioassays were used to determine the sediment quality four years after an oil spill (Prestige, 2002): the Microtox® test, a 10-day bioassay using the amphipod *Ampelisca brevicornis*, and a polychaete 10-day toxicity test with the lugworm *Arenicola marina*. In addition, bioaccumulation of PAHs was examined in the polychaete after 10 days of exposure. The results obtained from the toxicity tests and bioaccumulation analyses were statistically compared to the sediment chemical data, in order to assess the bioavailability of the contaminants, their effects, and their relationship with the oil spill. The sediments studied were from two areas of the Galician Coast (NW Spain): the Bay of Corme-Laxe and the Cíes Island, located in the Atlantic Island National Park. The results point to a decrease in contamination with respect to previous studies and to the disappearance of the acute toxicity four years after the oil spill. However an important bioaccumulation of PAHs was detected in the organisms exposed to sediments

from Corme-Laxe, suggesting that despite the recovery of the environmental quality of the area, effects in the biota might be occurring.

*Keywords: PAHs, toxicity, bioaccumulation, Microtox, amphipod, polychaete*

## 1. Introduction

The information obtaining by integrating the chemical concentration in sediments and from specific sediment bioassays (toxicity and bioaccumulation) is considered at the present time the best approach to assess the sediment quality and to test the toxicity and bioavailability of chemical compounds in sediments to benthic organisms (Casado-Martínez et al., in press; DelValls and Conradi, 2000; Chapman et al., 2002). Chemical analyses performed alone in bulk sediment do not always reflect the toxic fraction since they vary depending on the bioavailability and bioreactivity of the toxic compounds. An approach to this information can be reached by running bioassays.

The use of the commercial bioassay Microtox® has increased in recent years, due to the straightforward way to detect the “hot spots” of field contamination in the screening procedure (Mowat and Bundy, 2001; Stronkhorst et al., 2003; Van Beelen, 2003, Casado-Martinez et al, 2006a). Microtox® has also been used successfully in the past, to assess the impact of oil spills and oil contaminated sediments (Brohon et al., 2001; Kenneth et al., 2003; Pelletier et al., 2004; Morales-Caselles et al., 2007). Previous studies have shown that the amphipod specie *Ampelisca brevicornis* is a sensitive organism valid to assess toxicity of contaminated sediments (Riba et al. 2003; Casado Martinez et al., 2006b; Morales-Caselles, et al. submitted). The toxicity test according to standard methodologies (ASTM 1993) has been used in previous studies for the assessment of spills (Grant and Briggs, 2002; Briggs et al., 2003). The 10-d *Arenicola* bioassay (ASTM 1993) is relevant in PAH accumulation studies

because *A. marina* is a bulk sediment feeding polychaete worm that lives in a U-shaped burrow and is considered an indicator of PAH pollution (Rust et al., 2004b, Casado-Martínez et al., in press).

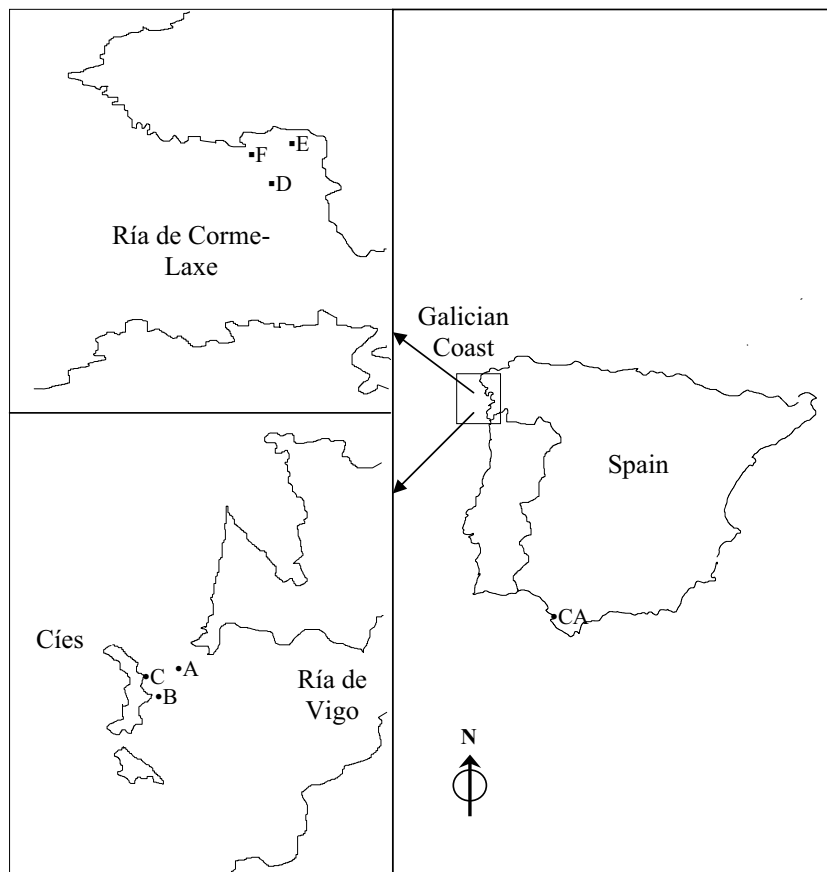
In November 13<sup>th</sup> 2002 the oil tanker *Prestige* suffered an accident 30 miles off the Galician Coast (NW Spain). Few days later, the tanker sank 150 miles offshore releasing approximately 60 000 tonnes of heavy fuel oil into the surrounding waters, which contaminated more than 1000 km of coastline. The physicochemical characteristics of the fuel oil from the tanker were similar to those from the *Erika* (France, 1999) (CSIC, 2003) ) and showed a low soluble and slow kinetics of degradation under natural conditions, so that it was expected to be accumulated in sediments (DelValls 2003). This paper presents the first attempt to assess the sediment contamination, toxicity and bioavailability of PAHs in the coast of Galicia four years after the sinking of the tanker *Prestige* (2002).

## **2. Material and methods**

### **2.1. Sampling**

After the accident of the tanker *Prestige*, the Cíes Island in the Atlantic Islands National Park in Galicia acted as a natural barrier protecting the coast. The Bay of Corme-Laxe, considered an area which develops farming, fishing, and shell fishing activities (Blanco et al., 2006) was also importantly affected by the spill. Surface sediments were collected in 2005-2006 with a 0.025 m<sup>2</sup> Van Veen grab near the island of Cíes Island in the Atlantic Islands National Park (stations A, B and C) in the 'Bay of Corme-Laxe' (stations D, E, F), and in the Bay of Cádiz (CA), the latter used as reference station (Figure 1). Sediment samples were kept in a cooler (4°C) and then transferred to the laboratory. Each

sediment sample was homogenized with a Teflon spoon until no colour or textural differences could be detected, and afterwards subsampled for physical characterization, chemical analysis and toxicity tests. Sediment toxicity tests were run no longer than 2 weeks after sampling.



**Figure 1.** Map of the coastal area of Galicia showing the locations of the sampling stations. A, B and C refers to the stations located in the Cíes Island in the Atlantic Island National Park and D, E and F to those in the Bay of Corme-Laxe. The station CA located in the Bay of Cadiz corresponds to the sediment used as negative control.

## 2.2 Microtox® test

In the commercial Microtox® test the decrease of the bioluminescence of the bacteria *Vibrio fischeri* is used as a quality indicator of the sample exposed. Measures of the IC50 (which is the dry sediment concentration that provokes 50% inhibition of the light emitted by the bacteria) provide the information about the sediment toxicity. The bioassay of bioluminescence inhibition with the bacteria *V. fischeri* was conducted on solid phase with the commercial Microtox® apparatus (model 500), by following the protocols for the basic solid phase test (BSPT), according to the standard operating procedure (AZUR Environmental, 1998) with the modifications reported by Campisi et al. (2005) and Casado-Martínez et al. (2006a).

## 2.3. Amphipod toxicity test

Individuals of the specie *Ampelisca brevicornis* used in the bioassay were collected by sieving the sediment in the field through a 0.6 mm mesh, as reported by Riba et al. (2003) and Casado-Martínez et al. (2006b). The collected biota were immediately transported to the laboratory where they were placed in 11-L aquariums with clean seawater and sieved sediment from the same location. Aeration was provided and natural photoperiod was selected. During acclimation water was replaced twice a week, and organisms were fed with special food for invertebrates (mixture made of aminoacids and organic particles).

The sediments were filtered (1 mm) prior to the toxicity test, in order to remove inorganic and organic debris and benthic organisms capable of preying *Ampelisca brevicornis*. The toxicity test was performed by exposing individual amphipods to their respective sediment from the different study sites. The percentage of survival after 10 days of exposure was the measured end point.

200 g of sediments were placed in 2 L glass beakers and about 800 mL of clean seawater was added. When the sediment settled down in the beakers, aeration was provided, and 12 hours afterwards the individuals were sieved from the acclimatization aquariums and 20 adults (3-5 mm) of *Ampelisca* were placed in each replicate. No food was provided during the experiment. The containers were kept in an incubator with photoperiod 12h-light/12h-dark and maintained at  $19 \pm 1$  °C during the 10 days of exposure. After this time, the beakers were sieved and the survival was counted in each replicate.

#### 2.4. Polychaete bioassay

The *A. marina* lugworms were sampled in the field by hand-digging and immediately transported to the laboratory in containers with sea water. Once in the laboratory, lugworms were placed in aquariums with sieved sediment from the survey area (5 cm thick) and acclimated for 7 d; air was provided and water was replaced three times per day. Water temperature was kept at 18 °C and natural photoperiod was selected. Filtered sediments from the study sites were placed in replicates in 11 L tanks reaching a 5 cm thick layer and clean sea water was added. Lugworms were put into the tanks (6 per tank) which were covered to avoid evaporation. Mortality was recorded daily and after 10 days of exposure surviving organisms were transferred to aerated clean sea water without sediment, where they were held for approximately 8 hours to induce gut emptying. Organisms were then frozen at -20 °C for PAHs bioaccumulation analysis.

#### 2.5. Chemical analysis

Geochemical matrix characteristics were examined by analyzing total organic carbon concentration and sediment grain size. Organic carbon content was determined using the method of Gaudette et al. (1974) with El Rayis' (1985)

modification. For sediment grain size, an aliquot of wet sediment was analyzed using a laser particle size Frisch (model Analysette 22), following the method reported by DelValls et al. (1998).

Trace metal analysis were analyzed as described by Casado-Martínez et al. (2006c); briefly, 2.5 g of sediments (<0.065 mm) were placed in Teflon containers and were digested in microwave (400W, 15 min, twice) with HNO<sub>3</sub> 2N in order to extract the fraction of metal with the greatest potential to be bioavailable. The extracts were purified by passing through a C-18 column and metals analyses were performed by anodic voltamperimetry (-Zn, Cd, Pb, Ni, Co and Cu- Metrohm Application Bulletin N° 147; - V- Metrohm Application Note N° V-81). For Hg the cold vapour technique was used and was quantified using atomic absorption spectrometry. The analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) and showed a recovery greater than 90% of the certified concentration. The reason why metals were analyzed in sediments is because we are studying environmental samples which may present a mixture of contaminants and this information may help to elucidate if the biological effects are due to other contaminants apart from PAHs.

To analyse polycyclic aromatic hydrocarbons in dried sediments and lugworms, samples were soxhlet extracted with *n*-hexane for 18 h, and with acetone /hexane (1:1) during 24h, respectively. Before extraction of organisms, surrogate PAHs standards were added to each sample (acenaphthene-d<sub>10</sub>, phenanthrene-d<sub>10</sub>, chrysene-d<sub>12</sub> and perylene-d<sub>12</sub>) were done. Compounds were isolated from extracts by column chromatography on Florisil-alumino-silica for sediments or silica-alumina for lugworms. PAHs were eluted with

dichlorometane/hexane (9:1 and 4:1). Aromatic fractions in sediments were analyzed on a Hewlett–Packard (HP) 5890 Series II gas chromatograph coupled with HP 5970 mass spectrometer. PAH concentrations in organisms were determined in a GC-MS Finnigan Mat GCQ TM in the selected ion mode. Chromatographic analysis was achieved with a 30 m × 0.250 mm DB-5 capillary column (0.25 µm film thickness) with helium as carrier gas. Injection was performed in the splitless mode at 280°C. Helium was used as carrier gas with a flow of 1 ml min<sup>-1</sup>. The oven temperature was programmed as follows: initial temperature was 75° C and then ramped to 130°C at 20°/min and then ramped to 320°C at the rate of 4°C/min and held for 15 minutes. The electron impact ionization mode conditions were the following: ion energy 70 eV and ion source and transfer line temperatures 220°C. PAH were identified by retention time and one characteristic mass fragment ion. Quantification of 16 PAH (acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[e]pyrene, perylene, indeno[1,2,3-cd]pyrene, dibenz[a,h]anthracene and benzo[g,h,i] perylene) were done using a 9-point calibration curve for each compound. Quality control samples were analyzed in each batch of 12 samples, reference material NIST, SRM 2977 (mussels), NRC-CNRC HS-6 (sediments) and blanks. The analytical procedure showed a recovery greater than 90% for sediment materials and between 75-125% for NIST material. Detection limits ranged from 1.0 to 5.0 ng g<sup>-1</sup>, dry weight, for organisms and for sediments.

## 2.6. Statistical analysis

Results obtained from the bioassays and the chemical measurements were linked by factor analysis, using principal components analysis (PCA) as the extraction procedure, which is a multivariate statistical technique for

exploring variable distributions (Riba et al., 2003). The objective of PCA is to derive a reduced number of new variables as linear combinations of the original variables. This provides a description of the data structure with the minimum loss of information. ANOVA was performed in order to determine significant differences ( $p < 0.05$ ) in survival organisms between the toxicity results obtained for the reference site and the other sampling sites.

### **3. Results and discussion**

#### **3.1. Chemical analysis**

Table 1 presents the general characteristics (fine particles and organic carbon) and the levels of PAH and trace elements in sediments from three groups of stations: Reference site (CA), Atlantic Islands National Park (A, B and C) and Corme-Laxe (D, E and F). Sediments consisted of large proportion of sand with low organic carbon content and diminished metal concentrations. Only Zn presented moderate concentrations in stations A and F. The levels of PAHs varied broadly among the stations: below detection limit in CA and C and higher values in A ( $108 \text{ mg kg}^{-1}$ ) and F ( $323 \text{ mg kg}^{-1}$ ). The comparison of PAH levels in 2005-06 with sediments collected in 2003-04 (Morales-Caselles et al., 2007) and 2004-05 (Morales-Caselles et al, 2006) showed a general decrease in concentrations in the stations surveyed at AINP and Corme-Laxe (Table 2). This comparison suggests a decrease of PAHs in the sediments of the two areas affected by the Prestige oil spill.

#### **3.2. Microtox® test**

Figure 2 shows the IC50 results obtained through the Microtox® test with the sediments collected in the three areas in 2005-06. All IC50 results were

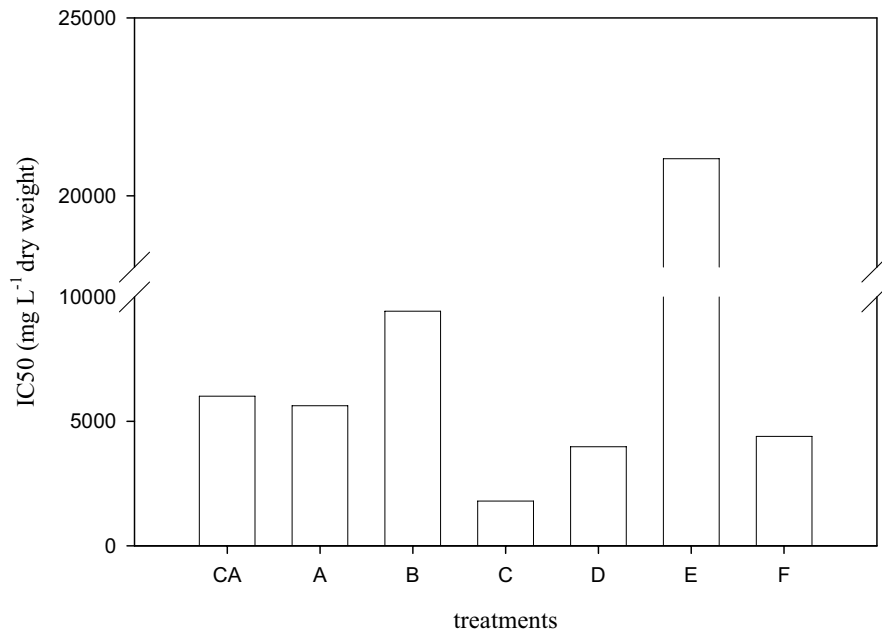
**Table 1.** Concentration of PAHs ( $\mu\text{g kg}^{-1}$  dry weight), metals ( $\text{mg kg}^{-1}$  dry weight), organic carbon (%dry weight) and percentage of fine particles ( $< 63 \mu\text{m}$  of dry sediment) in sediment samples. Reference: CA; Atlantic islands National Park: A, B and C; Corme-Laxe: D, E and F. n.d. = not detected value ( $< 0.005 \mu\text{g Kg}^{-1}$ ).

		PAH	Zn	Cd	Pb	Cu	Ni	Co	V	Hg	OC	fines
<b>REFERENCE</b>	<b>CA</b>	n.d.	21.3	0.9	2.3	7.0	0.1	3.4	80	0.05	1.1	1.0
<b>AINP</b>	<b>A</b>	108	377	0.3	1.5	5.2	13.3	0.3	0.7	0.04	0.4	4.3
	<b>B</b>	67	91	0.2	0.9	1.4	2.4	0.2	0.8	0.28	0.4	2.8
	<b>C</b>	n.d.	164	n.d	0.9	1.4	4.5	0.1	0.6	0.09	0.3	2.8
<b>Corme-Laxe</b>	<b>D</b>	38	25	0.2	3.7	0.7	1.7	0.3	2.0	0.16	0.3	3.8
	<b>E</b>	52	19.9	0.1	7.3	0.4	1.5	0.4	2.1	0.08	0.4	5.5
	<b>F</b>	323	271	0.2	5.9	4.2	5.7	0.4	3.4	0.08	0.7	6.0

**Table 2.** Table 2. Concentration of PAHs ( $\mu\text{g kg}^{-1}$  dry weight) in the following years after the *Prestige* oil spill (November, 2002). n.a: not available data; n.d.= not detected values ( $< 0.005 \text{ mg kg}^{-1}$ )

station	2003-2004	2004-2005	2005-2006
<b>A</b>	390	119	108
<b>B</b>	2120	366	67
<b>C</b>	420	239	n.d.
<b>D</b>	n.a	537	38
<b>E</b>	n.a	558	52
<b>F</b>	n.a	820	323

clearly above the limits assumed for sediment toxicity (expressed on dry weight units): 1000 mg L<sup>-1</sup> (Canadian Standards in Environment Canada, 2002) and 750 mg L<sup>-1</sup> (Spanish Standards in DelValls et al., 2004; Casado-Martínez et al., 2006). These results indicate that the tested sediments had no acute toxicity.

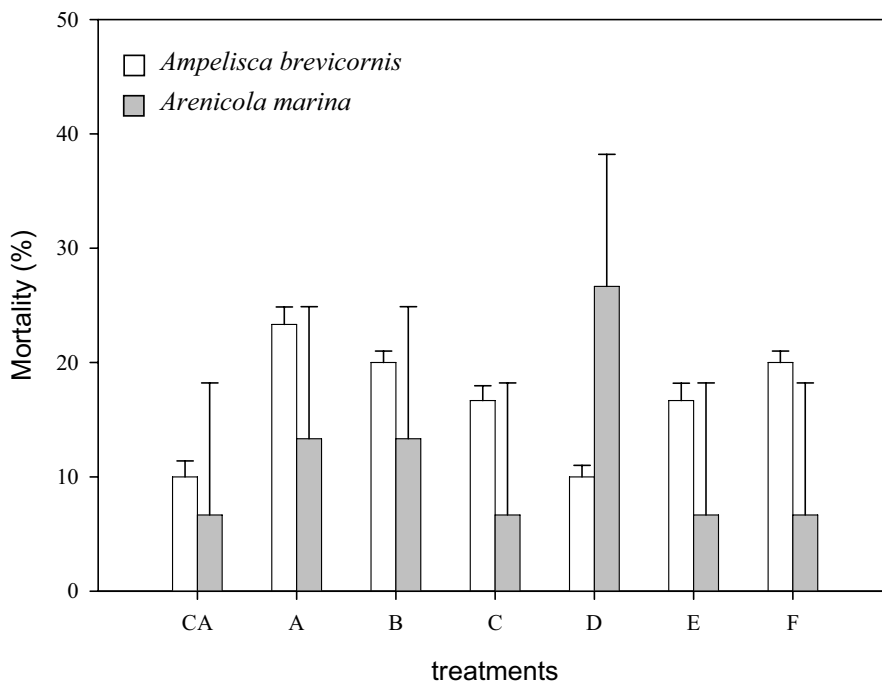


**Figure 2.** IC50 results obtained from the application of the Microtox<sup>®</sup> test to sediment samples from the various stations. The line indicates the limits below which the sediment sample is considered toxic by the Canadian Standards (1000 mg L<sup>-1</sup> dry weight).

### 3.3. Amphipod and polychaete toxicity tests

The results of the 10-d bioassays with amphipods and polychaete are given in Figure 3. Mean values of mortality was less than 25 and 27% for *Ampelisca* and *Arenicola* bioassays, respectively. None of the tested sediments were significantly ( $p < 0.05$ ) different from the reference sediment (CA), suggesting that sediments are not toxic in accordance with the US

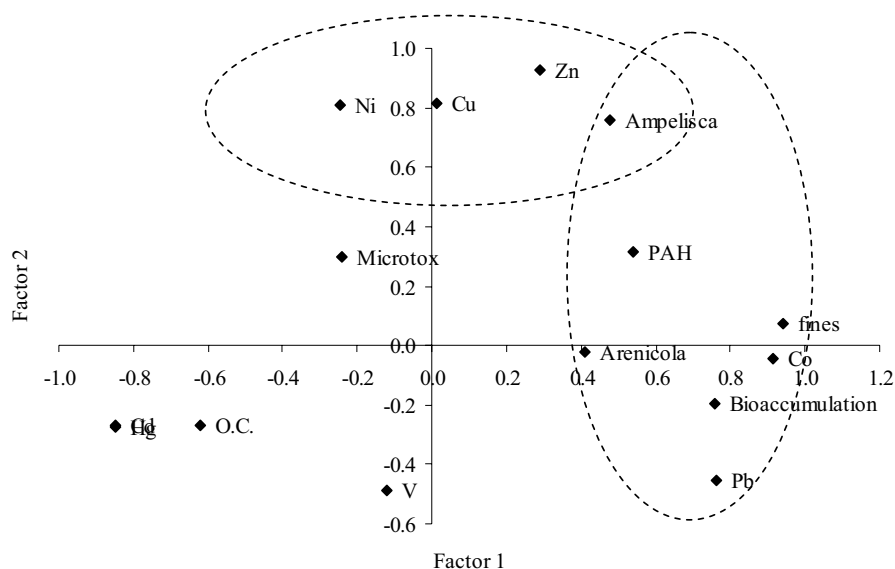
Environmental Protection Agency (USEPA, 1994) and guidelines reported by Casado-Martínez et al. (2006b). A high variability in the mortality results was observed in the polychaete assay and no correlation was observed between the amphipod and polychaete mortality results, which can be due to the different susceptibilities. This fact highlights the importance of using different organisms in this kind of studies, especially where diverse known and unknown contaminants are present and synergic/antagonic effects may occur.



**Figure 3.** Averaged mortality results and standard deviations after 10 days of exposing *Ampelisca brevicornis* and *Arenicola marina* to the sediment samples.

### 3.4. Bioaccumulation assay

Despite the absence of acute toxicity, lugworms exposed 10 days to sediments from the Galician Coast showed a higher accumulation of PAHs than those exposed to sediments from the reference station. The concentrations of PAHs in those worms exposed to sediments varied from 3.2-3.9 mg kg<sup>-1</sup> (Corme-Laxe) to 2.6-2.7 mg kg<sup>-1</sup> (Atlantic Island National Park). A PAHs bioaccumulation index  $BI = C_x/C_R$  was defined by the ratio between the PAH concentration in the lugworm exposed to sediment X ( $C_x$ ) and to the reference ( $C_R$ ). The BI ranged within the narrow interval of 1.0-1.6, meaning that lugworms presented a narrow interval of accumulation than sediments.

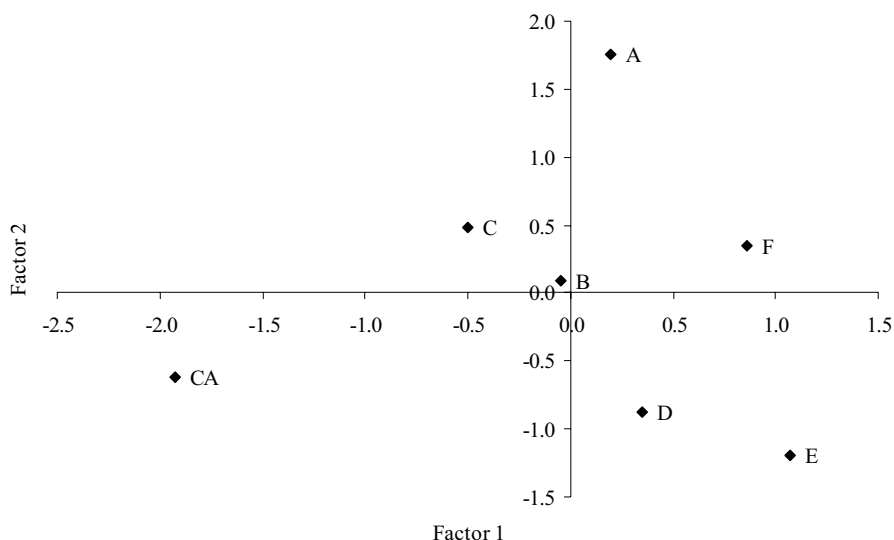


**Figure 4.** Factor loadings of 15 variables for the two principal factors resulting from the multivariate analysis of results obtained from the chemical analysis, the acute toxicity tests and the bioaccumulation assay.

### 3.5. Principal component analysis

A principal component analysis (PCA) was performed including sediment chemical parameters, toxicity tests and PAHs bioaccumulation index. The 15 variables can be grouped in 2 new factors which explain a 61.6 % of the total variance. Figure 4 represents the loadings of the variables in each factor. For a good association between variable and a factor we decided to interpret a group of variables as those associated to a particular component where the loading was 0.50 or higher which approximates to Comreys' cut-off of 0.55 (Comreys, 1973). The first factor, Factor #1, accounts for 39.0 % of the total variance and links the bioaccumulation of PAHs in the polychaete with the presence of PAHs, Pb, Co, and fines in the sediments. Factor #2 accounts for 22.6 % of the variance and shows the relationship between metals Zn, Cu and Ni with the low toxicity (<25% of mortality) to the amphipod *Ampelisca brevicornis*.

Figure 5 shows the influence of each factor in the 7 study sites. Factor #1, defined as the PAHs bioaccumulation due to their presence in sediments and the association with Pb and Co; it has mainly prevalence in the sites D (0.35), E (1.07) and F (0.86) from Corme-Laxe. This means that these stations present a contamination by PAHs which are able to bioaccumulate in the biota and are accompanied by the metals Pb and Co. However it does not reflect an acute toxicity of the benthic organisms exposed to the sediments. Despite the low mortality with the 10-d bioassays (Figure 3) the PCA suggests a relationship between sediment quality, bioaccumulation and the slight mortality. Factor #2, which relates a slight toxicity with the metals Zn, Cu and Ni bound to sediments, presents a positive loading in the sites A (1.75), C (0.49) and F (0.34).



**Figure 5.** Estimated factor scores for the two factors in each of the 7 studied cases. The factor scores quantify the prevalence of each factor for every station and are used to establish the definition of each factor.

### 3.6. General discussion

Chemical analyses of surface sediments from the Galician coast (Ría de Corme-Laxe and Cíes) indicate a substantial decrease in the content of PAHs four years after the oil spill. The effect of the oil spill from Prestige probably diminished due to biotransformation and volatilization of compounds in the sediments (Albers, 2003). It should not be ignored that hydrodynamic processes, namely sediment resuspension, may have influence in those processes (Neff, 2002). In addition, enhanced levels of V and Ni, which are often associated with hydrocarbon spills, suggests that their presence could be related to the *Prestige* oil spill. Additional sources could contribute to the amount of Zn and Cu found in these areas (Cobelo-García et al., 2004).

Apparently, the link between the results of the 10-d bioassays and the presence of PAHs in the sediments from Corme-Laxe is indicative of sediment toxicity. This link is however insufficient to describe those sediments as toxic, since values were lower than the international guidelines employed for this test. Moreover, the results of the Microtox test were unrelated. Despite the decrease with the time (Table 2) of PAH concentrations in sediments from the Galician area affected by the oil spill (Fernández et al., 2006; Soriano-Sanz, 2006; Morales-Caselles et al., 2007; Morales-Caselles et al., accepted), PAHs accumulation was identified in the organisms exposed to 10-d tests. Residues still remain in tissues of benthic organisms, probably indicating that PAHs are available to the food chain, and thus representing a potential risk to the wellbeing of the ecosystem. The fact that no acute toxicity was detected in the sediments and the bioaccumulation produced by the PAHs in Corme-Laxe (site E and F) suggests that there has been a recovery of the area affected by the accidental oil spill. Similar results were obtained years after major tanker spills, such as the *Exxon Valdez* (USA, 1989) (Lee and Page 1997), took place. However, previous studies have shown the relationship between the *Prestige* oil spill and sublethal effects in the organisms exposed to contaminated sediments from the Galician area (Martínez-Gómez et al., 2006; Marigómez et al., 2006; Morales-Caselles et al., 2006; Fernández et al., 2006; Soriano-Sanz, 2006).

#### **4. Conclusions**

The results obtained in the present study suggest that there has been a recovery of the quality of the sediments affected by the *Prestige* oil spill in the Galician Coast. Sediments from Corme-Laxe and the AINP do not present acute toxicity although the presence of some metals and PAHs in the sediments is considered a potential risk in those areas; even though PAHs do not tend to

bioaccumulate along the trophic chain (Neff, 2002), the accumulation of PAHs in the polychaete *Arenicola marina* was related to the presence of this contaminant in the Bay of Corme-Laxe and suggests the possibility of producing sublethal toxic effects to the organisms exposed despite there was not acute toxicity detected. Further studies are required, in order to follow up the sublethal effects years after the oil spill.

## **5. Acknowledgments**

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## Capítulo 3.

### **Estudio de efectos subletales en organismos bajo condiciones de laboratorio**

La importancia de los test de toxicidad a nivel subletal radica en que este tipo de estudio permite evaluar con una mayor sensibilidad la respuesta de un organismo ante un tipo de contaminación además de discriminar puntos con una contaminación moderada identificando los efectos subletales. Antes de que se produzca la muerte o la enfermedad, tanto organismos como poblaciones responden al estrés alterando diferentes parámetros a nivel molecular, histológico, inmunológico y fisiológico, a nivel de organismo, población o ecosistema (Livingstone, 1993; López-Barea, 1994). Los biomarcadores, permiten evaluar los efectos del estrés subletal sobre los organismos expuestos a sustancias contaminantes, reflejando el estado de los individuos a nivel molecular o celular como respuesta a dicho estrés. Además, estas medidas pueden identificar rápidamente la presencia de sustancias tóxicas, ofreciendo una alerta temprana antes de que las alteraciones lleguen a niveles de organización mayores. Los biomarcadores han mostrado ser las herramientas adecuadas para caracterizar el estado de los organismos presentes en zonas impactadas donde se da la presencia de mezclas complejas de contaminantes (Lafontaine et al., 2000; Munns et al., 2002; Galloway et al., 2004; Martín-Díaz et al., 2005, Montserrat et al., 2006).

Existen tres tipos generales de biomarcadores:

- Los *biomarcadores de exposición*, determinan, dentro de los organismos expuestos a contaminantes, metabolitos derivados de la biotransformación o productos de su reacción con moléculas biológicas.
- Los *biomarcadores de efecto*, muestran la respuesta del organismo expuesto al agente xenobiótico en particular o al complejo de mezcla.
- Los *biomarcadores a niveles de población, comunidad y ecosistema*, proporcionando información de las alteraciones a mayores niveles de organización.

Una vez realizados los ensayos agudos bajo condiciones de laboratorio tal y como se describe en el capítulo anterior, en el presente capítulo se recogen cinco trabajos en los que se discuten los resultados obtenidos en bioensayos de tipo subletal realizados bajo condiciones de laboratorio con cuatro especies marinas: el pez *Sparus aurata*, el cangrejo *Carcinus maenas*, la almeja *Ruditapes philippinarum* y el poliqueto *Arenicola marina*. Los ensayos subletales, complementan y mejoran la información obtenida por los experimentos agudos y fueron realizados en periodos de tiempo que varían entre los 15 y 60 días. En el primer artículo (V) se presenta un estudio de dos meses de duración en el que se llevaron a cabo exposiciones de *S. aurata* a sedimentos de la costa gallega afectados por el vertido del petrolero *Prestige*, y se midieron biomarcadores de exposición (actividad EROD y metalotioneinas) y efecto (histopatología), poniendo de manifiesto la importancia inicial del vertido. En el trabajo VI se realiza una valoración de la cinética de las enzimas implicadas en la detoxificación de PAHs (actividad EROD) en *S. aurata* y se relacionan con los daños sobre los tejidos causados por los contaminantes orgánicos. En el trabajo VII se muestran los resultados de un estudio realizado con *C. maenas* y *R. philippinarum* donde se expusieron los organismos a sedimentos de Galicia y

Algeciras. En este estudio se seleccionó una batería de biomarcadores de exposición relacionados con procesos de detoxificación (actividad EROD como biomarcador de la fase I y GST de la fase II) y actividad antioxidante (GPX, GR y FRAP). Estos biomarcadores se relacionaron con los contaminantes analizados en los sedimentos con el fin de identificar las sustancias causantes del estrés, entre las que destacaron los PAHs y algunos metales, principalmente en la Bahía de Algeciras y Corme-Laxe en Galicia.

**Tabla 3.1.** Relación de bioensayos subletales realizados para la evaluación de la calidad de los sedimentos.

Especie	Tiempo de exposición	Medida final
<i>Sparus aurata</i>	60 días	Actividad EROD, Metalotioneinas, Histopatología,
<i>Carcinus maenas</i>	28 días	Actividad EROD, GST, GPX, GR, FRAP, Vitelogenina, Histopatología
<i>Ruditapes philippinarum</i>	28 días	Actividad EROD, GST, GPX, GR, FRAP, Histopatología
<i>Arenicola marina</i>	15 días	alteración del comportamiento y alimentación, GR, GPX, GST, FRAP, TBARS, fagocitosis, daño de ADN

El trabajo VIII incluye un estudio de la variación de vitelogenina en el cangrejo *C. maenas* tras 28 días de exposición a sedimentos recogidos en Galicia y Algeciras. Los resultados confirman la relación de la variación de este biomarcador con contaminantes presentes en el sedimento, principalmente PAHs y algunos metales que varían en función de la zona de estudio, siendo la más afectada la Bahía de Algeciras. Este trabajo fue presentado en el congreso CEMEPE/SECOTOX 2007 y obtuvo el premio de mejor presentación oral de jóvenes científicos. Para finalizar este capítulo, el trabajo IX, realizado en gran parte durante una estancia en la Universidad de Plymouth (UK), presenta los

resultados obtenidos tras realizar exposiciones de quince días de duración con el poliqueto *Arenicola marina* y analizar un set de biomarcadores que incluyen: alteración del comportamiento y alimentación, enzimas implicadas en procesos de defensa (GR, GPX, GST, FRAP, TBARS y fagocitosis), efectos genotóxicos (daño de ADN). Cabe destacar que en este estudio se determinó por primera vez la capacidad de este invertebrado marino de activar procesos fagocíticos como respuesta a los contaminantes del sedimento. Las exposiciones se realizaron principalmente con sedimentos procedentes de la Bahía de Algeciras y el Parque Nacional de las Islas Atlánticas y los resultados de los biomarcadores se relacionaron con los contaminantes ligados al sedimento, mostrando, de nuevo, la degradación ambiental en la Bahía de Algeciras y la recuperación de las Islas Cíes cuatro años después del vertido.

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## Ecotoxicity of Sediments Contaminated by the Oil Spill Associated with the Tanker “Prestige” Using Juveniles of the Fish *Sparus aurata*

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**Abstract.** In November 2002, the oil spill from the tanker *Prestige* in the Galician Coast caused an ecological catastrophe in Spain. The adverse effects associated with the contaminants bound to sediments were tested using juveniles of the fish *Sparus aurata* (seabream). The approach evaluates sediment quality by using an integrated assessment including chemical and ecotoxicological data. Sediment samples were physicochemically characterized, and the concentration of contaminants (polycyclic aromatic hydrocarbons—(PAHs) and metals) was measured. Different biomarkers of exposure (metallothioneins and ethoxyresorufin *O*-deethylase activity (EROD)) and biomarkers of effect (histopathology) were analyzed along the time. A multivariate analysis approach was used to correlate concentration of contaminants and sublethal effects measured in individuals of fish. Results show that increasing concentrations of PAHs in sediments were related to increased EROD activities and histopathological lesions. This is the first evidence showing adverse effects associated with petroleum contamination of PAHs in sediments after this spill, and it demonstrates the utility of the sublethal toxicity tests for monitoring the impact of petroleum spills.

It has long been demonstrated that sediments can adsorb persistent and toxic chemicals to levels many times higher than water column concentrations (DelValls *et al.* 2002), whereas the sediment may become sufficiently polluted to disrupt natural biological communities (Adams *et al.* 1992; Tolun *et al.* 2001).

For a better assessment of the pollution process in the marine coastal environment, several authors have proposed determinations based on chemical measurements together with laboratory toxicity tests (Chapman 1988; Luoma and Ho 1992).

Sediment toxicity bioassays are instruments used to test the ecotoxicity and bioavailability of chemical compounds in sediments to benthic organisms. In this kind of bioassay, the organisms are exposed to sediment samples collected *in situ*

and after the incubation period, a biological response is measured; this response must be sensitive, ecologically relevant, and easy to standardize (Stebbing *et al.* 1980). Bioassays provide information on the toxicity of contaminated sediments that can be neither derived from chemical analysis nor from ecological surveys performed alone (Chapman and Long 1983; Long and Chapman 1985). Interest in the effects of environmental stressors on health and disease in fish and other marine organisms has increased in recent years, and in particular, histological and cellular alterations have been observed in marine fish from polluted coastal waters and estuaries (Malins *et al.* 1984; Stein *et al.* 1992). These sublethal responses have been found to be a powerful tool to evaluate sediment toxicity effects (DelValls *et al.* 1998a).

Biomarkers in fishes have been previously studied in the assessments of oil spills such as Exxon Valdez (Varanasi *et al.* 1995; Jewett *et al.* 2002), Braer (Ritchie and O'Sullivan 1994), and Sea Empress (Kirby *et al.* 1998). In the present study, histopathology was conducted as a biomarker of effect in order to measure the damage caused in the target tissues by the presence of chemicals in the sediments. Two biomarkers of exposure were selected to address the biological adverse effects associated with contaminants present in the studied sediments. The toxicity of metals was assessed by metallothionein (MT) induction, whereas ethoxyresorufin *O*-deethylase activity (EROD) represents a good marker in MFO (mixed-function oxygenase), which is the first mode of detoxification of many organic pollutants (polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls). The EROD measurement in fish is considered a monitor of pollution exposure and an indicator of potential future problems in the health of fish populations (Carballeira 2003). Furthermore, EROD induction can be documented in fish exposed to spilled petroleum despite low tissues of PAHs (George *et al.* 1995; Whyte *et al.* 2000); The reason for using metallothionein induction is because we are studying a mixture of contaminants in the environment, and metallothioneins are one of the main biomarkers. In addition, it has been proved that the induction of this biomarker is not always just related to metals (Stegeman *et al.* 1992; Muto *et al.* 1999; Van der Oost *et al.* 2003).

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The composition of the oil spilled by the tanker *Prestige* was a mixture of saturated hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes, with most of the PAHs being of medium to high molecular weight (Albaigés and Bayona 2003). Furthermore, it presents some trace metals such as Ni, V, Cu, Pb, and Zn (CSIC 2003; Albaigés and Bayona 2003; Prego and Cobelo-García 2003; Prego and Cobelo-García 2004). The physicochemical characteristics of this fuel show that the soluble fraction is low and the kinetics of degradation are slow under natural conditions so it is expected to be accumulated in sediments. The biological effects associated with the chemicals from the oil spill will be dependent on the nature of the ecosystem that accepts them and the organisms living in it (DelValls 2003). The first research notes about the early impact support the prediction that the acute toxicity of the weathered fuel (Mariño-Balsa 2003), very rich in high molecular weight compounds, was relatively low for the organisms tested (clams and microalgae). However, although concentrations of individual PAHs in aquatic environments are usually much lower than concentrations that are acutely toxic to aquatic organisms, sublethal effects can be produced (Albers 2003).

The results presented in this work show the status of the quality of the sediments 2 years after the accidental spill by linking sublethal responses measured in the fish exposed to oil-contaminated sediments with chemical data determined in sediments.

## Materials and Methods

### Approach

The present study was carried out by using sediment samples collected along different littoral areas in the North and the South of Spain. In the North, we chose sampling stations that have been affected by the oil spill in differing degrees and located along the Galician Coast (Ga1, Ga2, Ga3). Another sample was located in the South of Spain, in the Bay of Cádiz (BC) which is considered a pristine area (Riba *et al.* 2004a) and was used as the negative control reference. An artificial sample (TM) was made by mixing a toxic mud from an accidental mining spill in Spain (Aznalcóllar, April 1998) with the clean sediment and used as positive control (Riba *et al.* 2003).

Sediment samples from each station were collected with a 0.025-m<sup>2</sup> Van Veen grab and placed in a cooler until a sufficient amount of sediment was collected from a particular station (about 30 L). The contents of the cooler were homogenized with a Teflon® spoon until no color or textural differences could be detected. The samples were subsampled for physical characterization and chemical quantification. After that, sediment samples were maintained in the cooler at 4°C in the dark until used in sediment toxicity tests. Testing occurred within 2 weeks of collection. Sediment was filtered (0.5 mm) prior to the toxicity test in order to remove means interferences such as shells, predators, and other residues.

### Chemical Analysis

Sediment aliquots from each station were dried at room temperature prior to chemical analysis and then gently homogenized. Geochemical

matrix characteristics were studied analyzing total organic carbon concentration and sediment grain size. Organic carbon content was determined using the method of Gaudette *et al.* (1974) with El Rayis (1985) modification. For sediment grain size, an aliquot of wet sediment was analyzed using a laser particle size Frisch (model Analysette 22) following the method reported by DelValls and Chapman (1998b).

For trace metal analysis, the sediments were digested as described by Loring and Rantala (1992). Fe, Mn, Zn, and Cu concentrations in the extracts were determined with a Perkin-Elmer 2100 flame atomic absorption spectrophotometer. Concentrations of Hg and As were determined by means of Perkin-Elmer MHS-FIAS coupled with a Perkin-Elmer 4100 ZL spectrophotometer. The other trace metals were measured by graphite furnace atomic absorption spectrophotometry (Perkin-Elmer 4100 ZL). Results are expressed as mg kg<sup>-1</sup> dry sediment. The analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) and showed a 90–110 range.

PAHs were analyzed by using gas chromatography/mass spectrometry (U.S. Environmental Protection Agency SW-846 Method 8270); briefly, dried samples were Soxhlet extracted with *n*-hexane for 18 h, and the extracts were isolated by column chromatography on Florisil-alumino-silica. PAHs were eluted and their fractions were dried in a rotatory evaporator and redissolved in isooctane. Aromatic fractions were analyzed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph coupled with HP 5970 mass spectrometer. Chromatographic resolution was achieved with a 30 m × 0.250 mm DB-5 capillary column, which has a 0.25-μm film thickness, with helium as carrier gas. Quality control was carried out using NRC-CNRC HS-6 sediment reference material. The analytical procedure allows agreement with the certified values in a 90–112 range.

### Sediment Bioassays

Toxicity tests were carried out using juveniles of *Sparus aurata* obtained from an aquaculture farm and transported to the laboratory where the fish spent 1 month to acclimatize. *S. aurata* was selected because is a common species along the Spanish coast. Its biology is well known, having been used in previous pollution studies (DelValls *et al.* 1998a), and it is easy to acclimatize to laboratory conditions. The sea water used during the acclimatization period and the bioassay was clean marine water. A baseline of 10 randomly chosen individuals were weighed to provide data for feeding calculations. After the acclimatization period, the fish had a weight that averaged 4 ± 1 g.

Approximately 4 L of sediment from the negative control (BC) and the other stations (Ga1, Ga2, Ga3, TM) were placed in replicate 25-L glass tanks with clean sea water before the beginning of the experiment. After 24 h of particle settling, aeration was provided to maintain adequate oxygen concentrations (greater than 80% saturation). At the beginning of the test, another baseline group of 10 randomly chosen individuals was measured, weighed, anesthetized, and processed for biomarker responses (exposure and effect) to be used as the initial cellular control. Twelve individuals were placed in every tank after checking each tank's water quality and were fed 2 or 3 times per day with commercial food (approximately 0.2 g per fish per day of "Mar Perla T" 1.4–2.2 mm). The test was conducted over 2 months, during which time no mortality was recorded. After the exposure period, individuals from each station were anesthetized and processed for histopathological, MTs, and EROD analysis. During the experiment natural photoperiod was selected and constant temperature was maintained (19 ± 1°C). The physicochemical parameters pH, temperature, oxygen, and salinity were recorded and controlled when necessary to maintain quality control during the test. Water replacement was performed every day by renewing 33% of the water column using a peristaltic pump.

## Histological Procedures

Organisms from the toxicity tests were analyzed to determine the histopathological damages in different target tissues (liver and gills). When the water was renewed, the survival rate for all tanks was determined. Fish were removed from the tanks after 56 days of exposure time and samples were collected. Fish were anesthetized with 0.1% of 99% pure 2-phenoxyethanol during 5–10 min, then weighed, measured for length, and externally examined. Target tissues (liver and gills) from all of the organisms were obtained by dissection and then fixed in phosphate-buffered 10% formaldehyde (pH 7.2) for 24 h and embedded in paraffin. The histological sections were stained with hematoxylin–eosin and hematoxylin–VOF (Gutiérrez 1967). Sections were reviewed by light microscopy (Leitz Laborlux S) and photographed (Sony DKC-CM30). Damage to the tissues was semi-quantified by detecting the frequency of the lesions in each detected alteration.

## Biochemical Analysis

Fish were sampled for biochemical analysis, and after dissection, the liver was kept at  $-80^{\circ}\text{C}$  prior to the homogenization. The samples were homogenized following the procedure developed by Lafontaine *et al.* (2000).

## Metallothionein Concentration (MT)

Samples obtained to determine metallothionein content were centrifuged at 28,000g for 40 min. The supernatant was added to 0.9 ml of NaCl (0.9%), heated to  $95^{\circ}\text{C}$  for 4 min, and centrifuged at 10,000g for 15 min at  $4^{\circ}\text{C}$ . Supernatant was stored at  $-80^{\circ}\text{C}$  prior to MT concentration determinations by Anodic Stripping Voltammetry (Olafson and Olsson 1987) using purified rabbit metallothionein (Sigma-Aldrich). Total protein determination was carried out using the methodology described by Bradford (1976). Concentrations were expressed as  $\mu\text{g MT/mg}$  total protein.

## Mixed Function Oxidase Assay (EROD)

After homogenization of the samples, EROD samples were centrifuged at 10,000g for 30 min, and the supernatant was used for the EROD activity determination and the total protein content described by Bradford (1976). Mixed function oxygenase activity was measured using the adapted EROD assay (Gagné and Blaise 1993). Briefly, 50  $\mu\text{l}$  of supernatant (homogenate 10,000g for 30 min), 10  $\mu\text{M}$  7-ethoxyresorufin, and 10 mM reduced NADPH in 100 mM  $\text{KH}_2\text{PO}_4$  buffer (pH 7.4). The reaction was started by the addition of NADPH, was allowed to proceed for 60 min at  $30^{\circ}\text{C}$ , and stopped by the addition of 100  $\mu\text{l}$  of 0.1 M NaOH. The 7-hydroxyresorufin was determined fluorometrically using 520 nm (excitation) and 590 nm (emission) filters. 7-Hydroxyresorufin concentration in the samples was achieved through a standard calibration curve developed with concentrations of 7-hydroxyresorufin. Results were expressed as  $\text{pmol/mg}$  total protein.

## Statistical Analysis

Analysis of variance was performed in order to determine significant differences ( $p < 0.05$ ;  $p < 0.01$ ) among sites in relation to the bio-

markers responses; the Tukey test was used as the post-hoc comparison. Also, contamination and toxicity data were linked by factor analysis, and using principal components analysis (PCA) as the extraction procedure, which is a multivariate statistical technique to explore variable distributions (Riba *et al.* 2003). The original data set used in the analysis included two biomarkers (EROD activity and metallothionein induction), two histopathological indexes (lesions in gills (LIG), and lesions in liver (LIL)), the concentration of different contaminants (PAHs, Cd, Cr, Cu, Ni, Pb, Zn, Hg), and the geochemical matrix characteristics (including total organic carbon and grain size distributions). The objective of PCA is to derive a reduced number of new variables as linear combinations of the original variables. This provides a description of the structure of the data with the minimum loss of information.

## Results

### Sediment Contamination

Summarized results of total organic carbon, grain size (percent of fine grain  $<63 \mu\text{m}$ ), concentration of metals and PAHs are shown in Table 1. Of all the stations, the negative control (BC) showed the lowest values of most of contaminants. In general, it is observed that the concentration of PAHs in the area of Galicia ( $\text{Ga3} > \text{Ga2} > \text{Ga1}$ ) was higher than those measured in the toxic mud and the sediments from the Bay of Cádiz (not detected). The toxic mud, used as positive control, showed high levels of metals in comparison to the other sample sites.

These chemical data can be compared to international sediment quality guidelines (SQGs) that account for the chemical contaminants levels associated with biological effect (DeValls *et al.* 2004). In Table 1, the contaminants that exceed any SQG are highlighted. The letter that appears with the number indicates which SQG is surpassed.

### Biomarker Responses

The basal level of metallothioneins measured in liver on day 0 of exposure was  $20.1 \mu\text{g}^{-1} \text{mg}^{-1}$  and was lower than the levels of this biomarker after 56 days of exposure; the measures show that metallothionein levels in liver were significantly different ( $p < 0.5$ ;  $p < 0.01$ ) between fishes exposed to control and those exposed to other sediment (Figure 1). These differences were more significant for the station Ga3 and TM ( $p < 0.01$ ) than for the other two stations located in the area of Galicia, Ga1, Ga2 ( $p < 0.05$ ).

EROD activity determined in the liver of juveniles of *S. aurata* on day 0 was  $0.3 \text{ pmol mg}^{-1} \text{ min}^{-1}$ . Results after 56 days of exposure are higher than basal levels and showed low values in fish exposed to sediment samples with absence or low levels of total PAHs (TM and BC, respectively) and were significantly different ( $p < 0.05$ ) from stations affected to a different degree by the oil spill (Ga#) (Figure 1). The fish from these stations present high values of EROD activity and high levels of PAHs in their sediment (Ga1, Ga2, and Ga3). These relationships increase when the concentration of PAHs in sediments increases ( $\text{Ga3} > \text{Ga2} > \text{Ga1}$ ). The EROD activity in liver of fish exposed to sediments from station Ga2 and Ga3 showed a significant difference ( $p < 0.01$ ) between the control

**Table 1.** Values of total organic carbon (TOC) (% dry weight), fines (% dry weight), and the concentration of contaminants (polycyclic aromatic hydrocarbons (PAHs) and metals) in sediment samples (concentrations are expressed in mg kg<sup>-1</sup> dry weight)

	Contaminant	BC	Ga1	Ga2	Ga3	TM
PAHs (mg kg <sup>-1</sup> )	TOC	1.07	0.60	1.19	2.00	1.00
	Fines	1.04	0.06	0.03	0.01	10.1
	Total PAHs	n.d.	0.19	2.12	5.10 <sup>a</sup>	n.d.
	Fluorene	n.d.	0.08 <sup>a,c</sup>	0.13 <sup>a,c</sup>	0.35 <sup>a,d</sup>	n.d.
	Acenaphthene	n.d.	0.06 <sup>a,c</sup>	0.17 <sup>a,d</sup>	0.27 <sup>a,d</sup>	n.d.
	Naphthalene	n.d.	0.31 <sup>a,c</sup>	0.63 <sup>a,d</sup>	1.40 <sup>a,d</sup>	n.d.
	Phenanthrene	n.d.	0.10 <sup>a,c</sup>	0.15 <sup>a,d</sup>	1.36 <sup>a,d,e</sup>	n.d.
	Anthracene	n.d.	0.02	0.03	0.18 <sup>a,c</sup>	n.d.
	Fluoranthene	n.d.	0.12	0.18	0.10	n.d.
	Pyrene	n.d.	0.09	0.13 <sup>c</sup>	0.39	n.d.
	Benzo[a]anthracene	n.d.	0.05	0.09 <sup>c</sup>	0.20 <sup>c</sup>	n.d.
	Chrysene	n.d.	0.08	0.12 <sup>c</sup>	0.39 <sup>a,c</sup>	n.d.
	Benzo[fluoranthene]	n.d.	0.11	0.18	0.06	n.d.
	Benzo[e]pyrene	n.d.	0.08	0.13	0.16	n.d.
	Benzo[a]pyrene	n.d.	0.05	0.09 <sup>c</sup>	0.10 <sup>c</sup>	n.d.
	Perilene	n.d.	0.03	0.05	0.04	n.d.
	Dibenzo[ah]anthracene	n.d.	0.01 <sup>c</sup>	0.02 <sup>c</sup>	0.02 <sup>c</sup>	n.d.
	Indene[123-cd]pyrene	n.d.	0.02	0.02	0.02	n.d.
	Benzo[ghi]perilene	n.d.	0.01	0.02	0.06	n.d.
Metals (mg kg <sup>-1</sup> )	Cd	0.92	0.16	0.05	n.d.	5.40 <sup>a,d</sup>
	Cr	0.10	n.d.	2.00	1.51	3.28
	Cu	6.98	12.8	0.65	1.19	210 <sup>a,d</sup>
	Ni	0.06	1.71	0.42	0.66	8.50
	Pb	2.28	2.73	1.14	1.26	790 <sup>b,d,e</sup>
	Zn	21.3	14.7	3.95	6.45	2181 <sup>b,d,e</sup>
	Hg	n.d.	n.d.	0.01	n.d.	5.61 <sup>b,d,e</sup>

<sup>a</sup> ERL, Effect Range-Low (NOAA 1999).<sup>b</sup> ERM, Effect Range-Median (NOAA 1999).<sup>c</sup> ISQG, Interim sediment quality guideline (CCM 1999).<sup>d</sup> PEL, Probable effect level (CCM 1999).<sup>e</sup> SQG for San Francisco Bay (Long *et al.* 1989).

Notes: Not detected is expressed by n.d.

treatment. Also, those from station Ga1 were significantly different from control treatment but with different value of the statistical *p* (0.05).

For metallothioneins, Tukey test results set the five stations in three homogeneous groups according to the differences of averages among the sites. The first group includes only BC, which is the negative control; the second group is constituted by Ga1, Ga2, and Ga3; and the third group includes TM, which is the positive control.

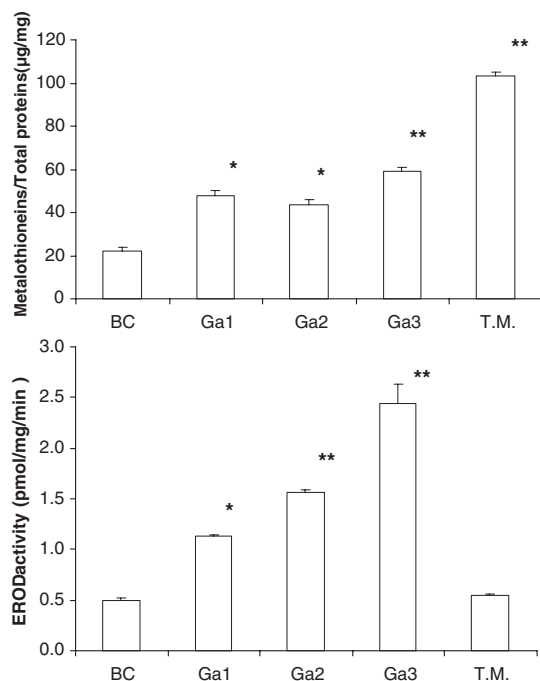
For EROD activity, there are three homogeneous groups set by the Tukey test results. The first group includes BC and TM, both negative and positive control, which do not present significant differences; the second group is constituted by Ga1 (*p* < 0.05) and Ga2 (*p* < 0.01); and the third group includes Ga3 (*p* < 0.01).

### Histopathological Approaches

The organisms analyzed on day 0 did not present histopathological damages. Different alterations were observed in target tissues (gills and liver) of fish exposed to sediment collected after 56 days of exposure in the different stations, mainly in gills, which showed shortening of secondary lamellae, hyper-

trophy, and hyperplasia, necrosis, and loss of epithelial cells in Ga# and TM; fusion of the secondary lamellae above all in Ga3 and TM; and presence of edematous areas in the distal portion of lamellae in Ga#. Also, liver showed lesions: vacuolization of hepatocytes, necrosis, and decrease of the zymogen granules of the exocrine pancreas in Ga# and TM. In general, an increase of cytoplasmic basophilia was detected in the liver and exocrine pancreas of all exposed fish related to the increase of PAHs. An example of some of these lesions is shown in Figure 2.

These lesions have been previously recorded as related to contaminants bound to sediments in *S. aurata* (DeIvals *et al.* 1998a) and in other fish species such as *Solea senegalensis* (Riba *et al.* 2004b, 2004c). As previously reported by DeIvals *et al.* (1998a) and based on the damage observed in the different tissues, histopathological alterations were evaluated semiquantitatively in the fishes exposed to the different stations by ranking the frequency of lesions measured in a total number of 6 individuals: – (0 individuals), +/- (1 individual), + (2 individuals), ++/+ (3 individuals), ++ (4 individuals), +++/++ (5 individuals), and finally the maximum is associated with the presence of a disease in the total number of individuals, +++ (6 individuals sampled). Gills were shown to be the most damaged tissue, showing different lesions mainly in Ga#



**Fig. 1.** Results of metallothionein concentration (mean and SD) in µg/mg of protein and EROD activity (mean and SD) in pmol/mg/min of protein in liver samples of *S. aurata* collected at 56 days of the experiment in sediments sampled in the Bay of Cádiz (BC), Galicia coast (Ga#), and toxic mud (TM) treatments. Asterisks indicate significant differences among the biomarker induction in the stations and the negative control (\*\* $p < 0.01$ , \* $p < 0.05$ )

and TM. An average of this semiquantitative evaluation of the frequency of the lesions measured from the different replicate results is shown in Table 2.

General indexes of lesion (lesion index in gills [LIG] and lesion index in liver [LIL]) were calculated for each tissue as an average value of the fish damage semiquantified (Figure 3).

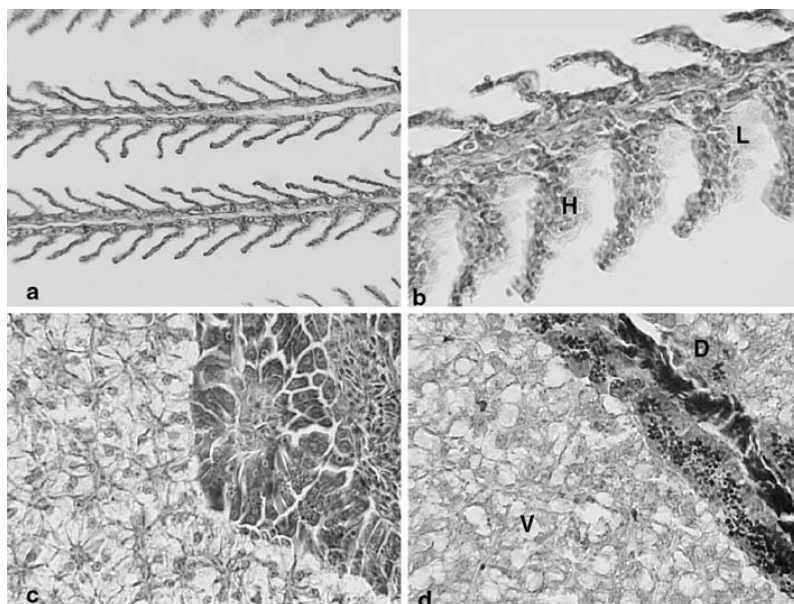
Fish exposure to sediment samples produced lesion damage related to the increase of the concentrations of contaminants (PAHs in Ga# and high levels of metals in TM) in the sediments selected in the bioassay. The lesions identified in all the tissues analyzed were almost always present in animals exposed to sediments from stations Ga2 and Ga3. Evaluations of histology of gills and liver revealed clear significant differences ( $p < 0.05$ ) between the negative control of toxicity and the Ga3 station and the toxic mud. The severity of the lesions detected in the tissues of fish exposed to sediments collected in Bay of Cádiz was lower than those measured in the area of Galicia. Results show the lowest indexes in the Bay of Cádiz (BC), which were significantly different ( $p < 0.05$ ) from the values from Galicia (Ga#) and toxic mud (TM). The index of lesions measured for gills (LIG) in Galicia increase with the presence of PAHs in the sediment samples (Ga3 > Ga2 > Ga1). The LIL results show that TM has the highest index related to liver lesions.

## Discussion

To link the set of data obtained, the original variables from chemical concentration and sublethal responses were analyzed by factor analysis, using PCA as the extraction procedure, which is a multivariate statistical technique (MAA) to explore variable (chemical concentration,  $n = 25$ ; toxicity data,  $n = 4$ ) distributions. The factor analysis was performed on the correlation matrix, and the variables were autoscaled (standardized) so as to be treated with equal importance (Riba *et al.* 2004a). The applications of MAA to the original 29 variables indicate that they can be grouped in two new factors. These factors explain 88.4% of the variance in the original data set. Negative values of sorted rotated factor loadings (negative salience) are as important as positive values (positive salience). In the present study, we selected to interpret a group of variables as those associated with a particular component where loading was 0.40 or higher (Table 3). This approximates Comreys' cutoff of 0.55 (Comreys 1973) for a good association between an original variable and a factor, and also takes into account discontinuities in the magnitudes of loadings approximating the original variables.

The first principal factor, #1, is predominant and accounts for 62.4% of the variance; it explains the toxicity of individual PAHs and combines the concentrations of PAHs (fluorene, acenaphthene, naphthalene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[fluoranthene], benzo[e]pyrene, benzo[a]pyrene, perylene, dibenzo[ah]anthracene, indene[123-cd]pyrene, benzo[ghi]perylene) in sediment, the total organic carbon, all the indexes of histopathological lesions (gills, LIG; and liver, LIL), and the EROD activity. The second factor, #2, accounts for 26.1% of the variance; it explains the toxicity associated with the metals in sediment combining, with negative loading, the chemical concentrations of the metals Cd, Cr, Cu, Ni, Pb, Zn, and Hg with the grain size with all the indexes of lesions (gills, LIG; and liver, LIL) and with the induction of metallothioneins (MTs). Figure 4 shows the influence of both factors in the five different stations. Factor 1, with positive loading, is defined as the toxic responses of the fish to PAHs bound to sediments; thus, station Ga2 (0.44) and especially Ga3 (1.48) show the significant prevalence of this factor, whereas this factor does not affect station Ga1, and both controls positive (TM) and negative (BC). The definition of Factor 2 as the toxic responses of the fish to metals bound to sediment only has prevalence in the positive control (TM); the negative loading of the toxic responses and the metals concentration in sediment implies that the prevalence of these factors in the station is associated with negative factor scores. Furthermore, a linear relationship can be observed in the scores of this factor from BC to Ga3, which confirms the increase of toxicity when PAHs increase in sediments.

It is estimated that about 63,000 tons of heavy fuel oil were lost from the single-hull tanker Prestige. Although a large quantity of this fuel was collected and removed from the coast, a large amount likely settled down at the bottom of the sea covered with sediment reaching the littoral area of the Galician coast after the first months of the spill (Albaiges and Bayona 2003). In the present study, we have aimed to assess the impact of this enrichment in littoral sediments collected in different affected areas and 2 years after the oil spill using juveniles of



**Fig. 2.** Example of histological sections associated with contaminants bound to sediments used in the *Sparus aurata* sediment toxicity test. (a) Gills from fish exposed to referent sediment showing primary lamellae and secondary lamellae arising from these, parallel with them and perpendicular to the filament axis BC (H & E  $\times 10$ ). (b) Hypertrophy and hyperplasia of the secondary lamellae Ga3 (H & E  $\times 25$ ). (c) Liver from control fish showing the exocrine pancreas around the blood vessels. Parenchymatous distribution of the hepatocytes in cords around the sinusoids BC (H & E  $\times 25$ ). (d) Hepatocytes and exocrine pancreas alteration TM (H & VOF  $\times 25$ ). D: decrease of the zymogen granules; H: hypertrophy and hyperplasia; L: loss of epithelial cells; V: vacuolization of hepatocytes

**Table 2.** Frequency of lesions detected in microscopic abnormalities of individuals of juveniles of the fish *Sparus aurata* sampled in the Bay of Cádiz (BC), Galicia coast (Ga#) and toxic mud (TM) treatments on day 56 of exposure

Organ	Histopathology	Samples zones				
		BC	Ga1	Ga2	Ga3	TM
Gills	Hypertrophy/hyperplasia	+/-	+++	++	+++	+/++
	Fusion of secondary lamellae	+	+/++	++	+/++	+
	Shortening of secondary lamellae	+	+/++	+/++	+/++	+/++
	Edematous areas or aneurysm in distal portion of lamellae	+/++	+/+++	+/+++	+++	+/++
	Necrosis and lost of cells epithelial	+	+/++	+/++	++	++
Liver	Increase of lipid vacuoles in the hepatocytes	+/-	+	+	+	++
	Increase of cytoplasmic basophilia of hepatocytes	+/-	+	++	+	+/-
	Necrosis and decrease of the zymogen granules of exocrine pancreas	-	+/-	+	+/-	+

(0 individuals), +/- (1 individual), + (2 individuals), +/+ (3 individuals), ++ (4 individuals), +++/++ (5 individuals) and finally the maximum is associated with the presence of a disease in the total number of individuals, +++ (6 individuals sampled).

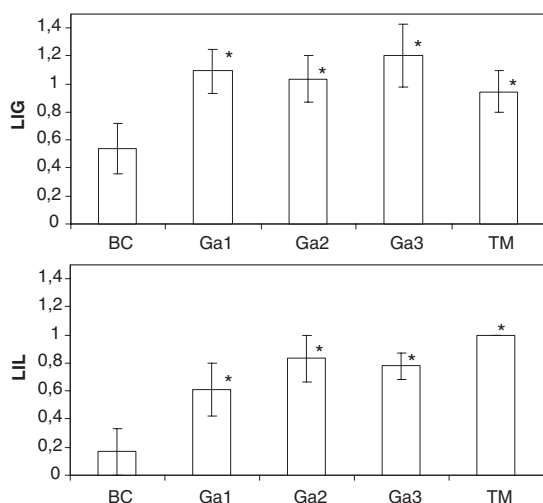
the fish *S. aurata* by means of different sublethal endpoints such as histopathological lesions, metallothionein induction, and EROD activity.

Previous studies have shown how in water the toxicity of individual PAHs increases as molecular weight (MW) increases up MW 202 and beyond it; solubility reduces and so does lethal toxicity, but sublethal effects can result (Albers 2003). In the present study, it has been shown that the Galician sediments (mainly Ga2 and Ga3) analyzed present levels of PAHs with low MW (fluorene, acenaphthene, naphthalene, phenanthrene, anthracene), medium MW (pyrene), and high MW (benzo[a]anthracene, chrysene, benzo[a]pyrene, dibenzo[a,h]anthracene), higher than some of the SQGs proposed by international agencies. Furthermore, all individual PAHs seem to have induced hepatic EROD and to produce histopathological damage; it is quite difficult to determine which of the individual PAHs is the main pollutant that has caused the biological effects (explained by factor 1 in the MAA);

however, it can be concluded that PAHs are the compounds that are producing the adverse effects to the fishes.

The significant differences of EROD induction between Ga# (Ga1,  $p < 0.05$ ; Ga2 and Ga3,  $p < 0.01$ ) show a strong relationship with the concentration of PAHs in the Galician sediments (Ga1, Ga2, and especially in Ga3)—impacted by the oil spill—and the histopathological lesions in gills and liver, studied in the MAA. Despite differences in the induction of EROD among these Galician samples, the validity of this biomarker of contamination was shown.

In the absence of fish mortality, other research on the impact of the “Sea Empress” oil spill in the UK in 1996 (Edwards and White 1999) showed the possibility of sublethal and chronic effects using a variety of techniques such as EROD activity. In these studies, there was evidence of high levels of EROD activity in the sites exposed to oil constituents in comparison with the control sites. There are other studies, carried out using biomarkers as EROD activity, that



**Fig. 3.** General indexes of lesions (mean and SD) measured in gills (LIG) and liver (LIL) of *Sparus aurata* juveniles exposed to sediments sampled in the Bay of Cádiz (BC), Galicia coast (Ga#), and toxic mud (TM) treatments. Asterisks indicate significant differences among the index value in the stations and the negative control (\* $p < 0.05$ )

support the conclusion of the persistent exposure of the organisms to hydrocarbons after 10 years of the oil spill caused by the tanker Exxon Valdez in Alaska in 1989 (Jewett *et al.* 2002), emphasizing the potential for continuing oil availability to biota. The ability of fish to metabolize many PAHs makes the use of EROD induction for biomonitoring purposes more beneficial than analytical measurements of PAH uptake, providing a sensitive chemical exposure information many years after a contamination event (Whyte *et al.* 2000). The histopathological analysis showed histomorphological alterations that have been previously reported in this organism when affected by sediment contamination caused by metals and organic compounds (DelValls *et al.* 1998a; Riba *et al.* 2004b, 2004c; Ortiz *et al.* 1999; Au 2004) such as hyperplasia and hypertrophy of gills, and alterations in hepatocytes and exocrine pancreas (*i.e.*, increased cytoplasmic basophilia and vacuolization, necrosis, loss of zymogen granules, *etc.*). An increase of cytoplasmic basophilia was generally detected in liver and exocrine pancreas of all exposed fish. This fact could be related to a decreased protein synthesis (Sarasquete and Gutiérrez 2005), and possibly related to necrotic focus. Moreover, loss of cytoplasmic hepatic glycogen is an early toxic response and may cause an apparent increase in cytoplasmic basophilia (Vethaak and Wester 1996). In general, contaminants can produce osmoregulatory, acid-base, or hemodynamic dysfunctions, and it was proposed that such symptoms are secondary to toxin interactions with specific transport steps or membrane-bound receptors (Evans 1987).

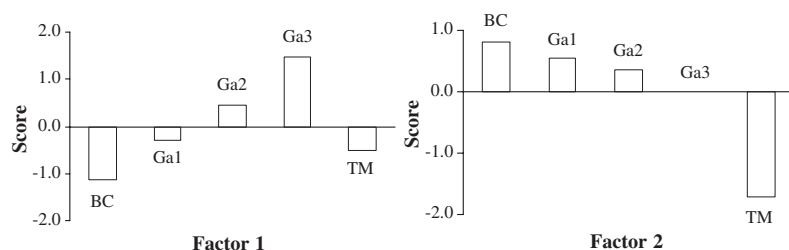
Results show that LIG is always higher than LIL in all of the stations; this could be explained by the affirmation that fish gill is a multifunctional organ sensitive to chemicals in water, because gill filaments and lamellae provide a very large sur-

**Table 3.** Sorted rotated factor loadings (pattern) of 29 variables for the two principal factors resulting from the multivariate analysis of results obtained from the bioassay with juveniles of *Sparus aurata*

% Variance	Factor 1 62.4	Factor 2 26.1
TOC	0.78	—
Fines	—	−0.93
Fluorene	0.96	—
Acenaphthene	0.97	—
Naphthalene	0.97	—
Phenanthrene	0.88	—
Anthracene	0.90	—
Fluoranthene	0.63	—
Pyrene	0.95	—
Benzo[a]anthracene	0.97	—
Chrysene	0.95	—
Benzo[fluoranthene]	0.43	—
Benzo[e]pyrene	0.93	—
Benzo[a]pyrene	0.92	—
Perilene	0.83	—
Dibenzo[ah]anthracene	0.91	—
Indene[123-cd]pyrene	0.83	—
Benzo[ghi]perilene	0.95	—
Cd	—	−0.91
Cr	—	−0.88
Cu	—	−0.95
Ni	—	−0.95
Pb	—	−0.96
Zn	—	−0.96
Hg	—	−0.96
MT	—	−0.97
EROD	0.98	—
IGG	0.79	−0.54
IGL	0.50	−0.74

face area for direct and continuous contact with contaminants in water. Fish gill and liver are highly sensitive to pollutant exposure; however, as previously indicated (Arellano *et al.* 1999), these pointed histopathological alterations are, in general, nonspecific effects, meaning that they are responsive to a variety of pollutants, and therefore only indicative of the general quality of the environment rather than specific types of pollutants (Au 2004). The increase of lipid vacuoles (small size) present in the hepatocytes can indicate an alteration of lipid metabolism or a partial change in their morphology, or in that of lysosomes (Arellano *et al.* 1999; Segner and Storch 1985). The cause-effect relationships and detailed mechanisms leading to the development of most pathological symptoms are not generally clear. Nevertheless, certain hepatic lesions in fish have been well correlated with contaminant exposure (Au 2004). Lamellar fusion of gills could be a protective effect for diminishing the amount of vulnerable gill surface area (Mallat 1985).

The comparison between chemical analysis and the different toxic response (biomarkers of exposure and of effect at different levels) is a useful tool to determine the quality of the studied sediments. The importance of the use of chronic bioassays that provide long-term information on the effects of the exposure to a toxic compound has been proved, because a compound cannot reflect a considerable lethal toxicity, but it is able to produce lesions at different levels to the organism exposed.



**Fig. 4.** Estimated factor scores for the two factors in each of the five cases. The factor scores quantify the prevalence of every factor for each station and are used to establish the description of each factor

Despite the repercussion of the spill in the biota, shown as a decrease of the abundance of the microfauna (Junoy *et al.* 2005), previous studies have shown that there was not an important toxic effect in different marine organisms (clams and microalgae) exposed to samples of the sediments and their elutriates associated with the spill caused by the tanker “Prestige” (Mariño-Balsa *et al.* 2003). The bioassay using juveniles of the fish *S. aurata* showed results sensitive enough to determine the hazard associated with this oil-contaminated sediment, displaying good correlation between the toxicity and the contaminant levels using a sublethal set of measurements including both biomarkers of exposure and effect. This study demonstrates the necessity to monitor the impact of the spill on sediment quality in the areas affected. Furthermore, it shows that a subchronic test using a sensitive and sublethal endpoint is a powerful tool to identify the risk associated with the enrichment of PAHs in affected sediments. The higher sensitivity of this bioassay compared to the acute tests previously used indicates the need to incorporate this kind of approach as part of a more complete and integrated study based on a weight-of-evidence approach, as previously recommended by some authors (Carballeira 2003).

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## Kinetic of Biomarker Responses in Juveniles of the Fish *Sparus aurata* Exposed to Contaminated Sediments

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**Abstract** Sediments in the National Park of the Atlantic Islands (Galicia, Spain) were affected by the spill of the tanker *Prestige* (November, 2002) and still present high levels of Polycyclic aromatic hydrocarbons. The adverse effects associated with the contaminants in sediments were tested using a chronic bioassay, exposing juveniles of the fish *Sparus aurata* (seabream). A toxicokinetic approach is proposed to evaluate sediment quality by linking chemical and

ecotoxicological data along the time. Sediment samples were physicochemically characterized and the concentration of contaminants (Polycyclic aromatic hydrocarbons – PAHs – and metals) was measured. Fishes were exposed to contaminated sediments, and samples from different tissues were collected every 15 days throughout the 60 days that lasted the experiment. A biomarker of exposure (ethoxyresorufin *O*-deethylase activity – EROD activity) and a biomarker of effect (histopathology) were analyzed during the exposure period. Results show a relationship between the biomarkers and the concentrations in sediments of polycyclic aromatic hydrocarbons—PAHs. Besides, the toxicokinetic approach links biomarkers response providing information about the relationship between the detoxification process and the damages observed in the different tissues. The frequency of the histological damage is highest when the EROD activity slightly decreases in accordance with the mechanism of detoxification of this enzymatic system against PAHs and other organic contaminants.

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### 1 Introduction

The heavy fuel oil spill from the tanker *Prestige* on November 2002 affected more than 1,000 km of coast, from the North of Portugal up to the South-east

of France, being the Galician Coast the most damaged. The composition of this fuel was a mixture of saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes, being most of the Polycyclic aromatic hydrocarbons—PAHs—of an intermediate-high molecular weight (Albaigés & Bayona, 2003; Blanco, Prego, Azpiroz, Fernández-Domínguez, 2006). The formation of the emulsions and the generation of tars induced processes of sedimentation, so that 1 year after the accident the marine sediments reached PAHs concentrations which were 10 times higher than those registered before the spill (IEO, 2003).

The use of biomarkers in fish which are indicative of PAHs exposure may provide an early warning of potential ecosystem degradation, contaminant bio-availability, and the defence responses of exposed organisms (Goksøyr et al., 1996; Goksøyr & Förlin, 1992; Reynolds et al., 2003). Interest in the effects of environmental stressors on health and alterations in fish and other marine organisms has increased in recent years, and in particular, histological and cellular alterations have been observed in marine fish from polluted coastal waters and estuaries (Malins et al., 1984; Stein et al., 1992). The capacity of many pollutants to alter different cells, tissues or organs has led to design histopathological techniques in order to evaluate the effects of contaminants (Lowe, 1988; Sarasquete, Muñoz-Cueto, Arellano, & González de Canales, 1997). On the other hand, the relation between contaminated environments and fish alterations has been proved by different authors (Ortiz, González de Canales, & Sarasquete, 2003; Husoy, Myers, & Goksøyr, 1996; Martín-Díaz, Tuberty, McKenney, Sales, & DelValls, 2005; Myers, Willis, Husoy, Goksøyr, & Collier, 1995; Ortiz, González de Canales, Sarasquete, 1999; Sarasquete et al., 2002).

The cytochromes P-450-1A (CYP1A) are of special interest in ecotoxicology, due to their role in the biotransformation and bioactivation of different organic xenobiotics (dioxins, PAHs, PCBs). The complex CYP1A turns by monooxygenation, determined lipophilic xenobiotics, in more water-soluble metabolites, helping its detoxification. The EROD measurement in fish is considered a monitoring instrument of pollution exposure and an indicator of potential future problems in the health of fish populations (Carralreira, 2003).

According to other authors (Moore & Simpson, 1992; Pacheco & Santos, 2002), the information provided by each biomarker individually is of limited

relevance, as there is a considerable likelihood of misinterpretation; thus, biomarkers are best used as selected batteries of tests rather than individually. Furthermore, the study of the behaviour of various biomarkers along the time (toxicokinetic approach) may lead to a substantial improvement in the knowledge of integrated fish toxic response (Pacheco & Santos, 2002).

In the present study a bioassay using the fish *Sparus aurata* was conducted by exposing the individuals to environmental sediment samples collected in areas affected by the *Prestige* oil spill (November 2002) in the National Park of the Atlantic Islands two years after the *Prestige* oil spill. The main objectives of this study were: (1) to characterize the metals and PAHs contamination in sediments from the selected areas in the Galician Coast and compare them to a pristine area in the Gulf of Cádiz; (2) to determine the sediment toxicity through the study of the two biomarkers selected along the time; (3) to determine and compare the sediment quality of the different areas of the study by linking the contamination data and the biological effects, establishing a mechanism of detoxification and proposing a toxicokinetic approach.

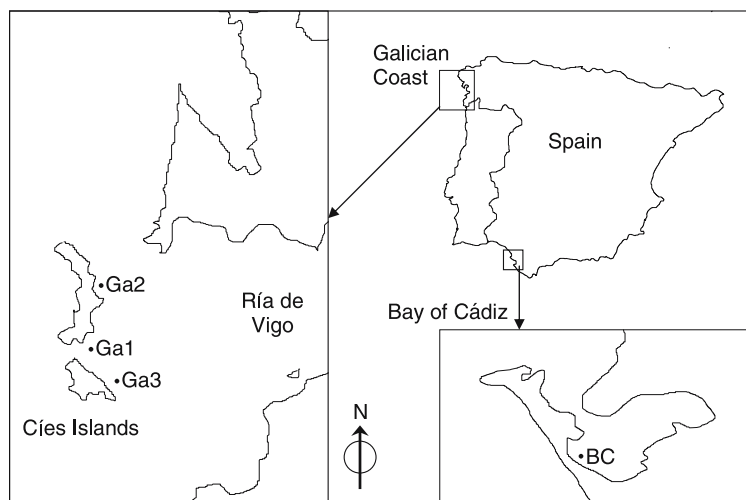
## 2 Material and Methods

### 2.1 Approach

The area selected to carry out this study was the “Cíes” islands located in the national park of the Atlantic Islands which has a high ecological value. These islands played an important role during the *Prestige* oil spill, since they operated as a natural barrier against the entry of fuel in the “Rías Bajas” (Galician Southern coast). Three stations (Ga1, Ga2 and Ga3), whose sediments were affected in different degree by the oil spill of the tanker *Prestige*, were selected in the internal face of the Archipelago (Figure 1). Another sample was located in the South of Spain, in the Bay of Cádiz (BC) which is considered a clean area (Riba, Forja, Gómez-Parra, & DelValls, 2004b) and was used as the reference station.

Sediment samples from each of the stations were collected with a 0.025 m<sup>2</sup> Van Veen grab and were homogenized with a Teflon® spoon until no colour or textural differences could be detected. The samples

**Figure 1** Map of the locations of the area selected to perform the study. *Ga1*, *Ga2* and *Ga3* are located in the Atlantic Islands in the Galician Coast affected by the oil spill related to the *Prestige* tanker (November, 2002), whereas the reference station (*BC*) is located in the Bay of Cádiz in the South of Spain (not affected by oil spills).



were subsampled for physical characterization and chemical quantification. After that, sediment samples were maintained at 4 °C in the dark until use in sediment toxicity tests (no more than 2 weeks). Sediment was filtered (1 mm) prior to the toxicity test in order to remove means interferences as shells, predators and other residues.

## 2.2 Chemical analysis

Sediment aliquots from each station were dried at room temperature prior to chemical analysis and then gently homogenized. Geochemical matrix characteristics were studied analyzing organic carbon (TOC) concentration and sediment grain size. For sediment grain size an aliquot of wet sediment was analyzed using a laser particle size Frisch (model Analysette 22) following the method reported by DelValls, Blasco, Sarasquete, Forja, and Gómez-Parra (1998). Organic carbon content was determined using the method of Gaudette, Flight, Torner, and Folger (1974) with El Rayis (1985) modification.

Sediments were digested for trace metal analysis, as described by Loring and Rantala (1992). Zn, and Cu concentrations in the extracts were determined with a Perkin-Elmer 2100 flame atomic absorption spectrophotometer. Cd, Cr, Ni and Pb were measured by graphite furnace atomic absorption spectrophotometry (Perkin-Elmer 4100 ZL), while concentrations of Hg were determined by means of Perkin-Elmer MHS-

FIAS coupled with a Perkin-Elmer 4100 ZL spectrophotometer. Results are expressed as mg kg<sup>-1</sup> dry sediment. The analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) and comply with the certified values in over a 90%.

Polycyclic aromatic hydrocarbons (PAHs) were analyzed by using a gas chromatography equipped with an electron capture detector (ECD) (U.S. Environmental Protection Agency SW-846 Method 8270) (US EPA, 1984); briefly, dried samples were soxhlet extracted with n-hexane for 18 h, and the extracts were isolated by column chromatography on Florisil-alumino-silica. PAHs were eluted and their fractions were dried in a rotatory evaporator and redissolved in isooctane. Aromatic fractions were analyzed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph coupled with HP 5970 mass spectrometer. Chromatographic resolution was achieved with a 30 m×0.250 mm DB-5 capillary column, which has a 0.25 µm film thickness, with helium as carrier gas. Quality control was carried out using NRC-CNRC HS-6 sediment reference material. The analytical procedures comply with the certified values in over a 90%.

## 2.3 Sediment bioassay

Juveniles of *S. aurata* were obtained in an aquaculture farm and were transported to the laboratory where the

fish spent one month to acclimatize before the bioassay. *S. aurata* was selected because is a common specie in the Spanish coast, its biology is well known, has been used in previous pollution studies (DelValls et al., 1998) and is easy to acclimatize to laboratory conditions. Sediment (approximately 4 l) from the negative control (BC) and the stations Ga1, Ga2, and Ga3 were placed in replicate in 25-l glass tanks with clean sea water before the beginning of the experiment. After 24 h of particle setting, aeration was provided to maintain adequate oxygen concentrations (higher than 80% saturation). A baseline group of 10 randomly chosen individuals were measured, weighed, anaesthetized, and processed for biomarkers responses (exposure and effect) to be used as the initial cellular control. After checking the tanks water quality, twelve individuals (with a weight averaged  $4 \pm 1$  g) were placed in every tank and were fed two or three times per day. The test was conducted during 2 months, no mortality was recorded, and every 15 days six individuals from each station were anaesthetized and processed for histopathological and EROD analysis. During the experiment natural photoperiod was selected and temperature was maintained constant ( $19 \pm 1$  °C). Physicochemical parameters (ammonia, pH, temperature, oxygen and salinity) were recorded and controlled when necessary to maintain quality control during the test. Water replacement was performed every day to avoid increasing levels of ammonia, and the survival rate for all tanks was determined.

#### 2.4 Histological procedures

Individual of the fish *S. aurata* proceeding from the toxicity tests were analyzed to determine the histopathological damages in gills. Fish were removed from the tanks at 15, 30, 45 and 60 days of exposure time and samples were collected. Fish were anaesthetized with 0.1% 2-phenoxyethanol 99% during 5–10 min; then weighed, measured in length and externally examined. Liver and gills from all the organisms were obtained by dissection and then fixed in phosphate buffered 10% formaldehyde (pH 7.2) for 24 h and embedded in paraffin. The histological sections were stained with Haematoxylin–Eosin and Haematoxylin–VOF (Gutiérrez, 1967). Sections were reviewed by light microscopy Leitz Laborlux S and photographed (Sony DKC-CM30).

General indexes of histological lesions were calculated for each tissue (lesion index in gills [LIG] and lesion index in liver [LIL]) as an average value of the fish damage semi-quantified as previously reported (Morales-Caselles, Jiménez-Tenorio, González de Canales, Sarasquete, DelValls, 2006; Riba, Casado-Martínez, Blasco, DelValls, 2004a; Riba et al., 2004b; Riba, González de Canales, Forja, & DelValls, 2004c). The semiquantification was performed by ranking the frequency of lesions measured in a total number of six individuals: – (zero individuals), +/- (one individual), + (two individuals), ++/+ (three individuals), ++ (four individuals), +++/++ (five individuals) and finally the maximum is associated with the presence of alterations in the total number of individuals, +++ (six individuals sampled).

#### 2.5 Biochemical analysis

Fish were sampled for biochemical analysis, and after dissection, livers were kept at  $-80$  °C prior to the homogenization. The samples were homogenized following the procedure developed by Lafontaine et al. (2000). After homogenization of the samples, EROD samples were centrifuged at  $10,000 \times g$  for 30 min, and the supernatant was used for the EROD activity determination and the total protein content described by Bradford (1976). EROD assay was performed following the methodology described by Gagné and Blaise (1993). Briefly, 50  $\mu$ l of supernatant (homogenate  $10,000 \times g$  for 30 min), 10  $\mu$ M 7-ethoxyresorufin and 10 mM reduced NADPH in 100 mM  $\text{KH}_2\text{PO}_4$  buffer (pH 7.4). The reaction was started by the addition of NADPH, being allowed to proceed for 60 min at 30 °C, and stopped by the addition of 100  $\mu$ l of 0.1 M NaOH. The 7-hydroxyresorufin was determined fluorometrically using 535 nm (excitation) and 580 nm (emission) filters. 7-Hydroxyresorufin concentration in the samples was achieved through an standard calibration curve developed with concentrations of 7-hydroxyresorufin. Results were expressed as picomoles per milligram Total protein (Martín-Díaz, 2004).

### 3 Results and Discussion

Table 1 shows the summarized results of total organic carbon, grain size (% of fine grain  $< 63$   $\mu$ m),

**Table 1** Values of total organic carbon (% dry weight), fines (% dry weight) and the concentration of contaminants (PAHs and metals) in sediment samples (concentrations are expressed in mg kg<sup>-1</sup> dry weight)

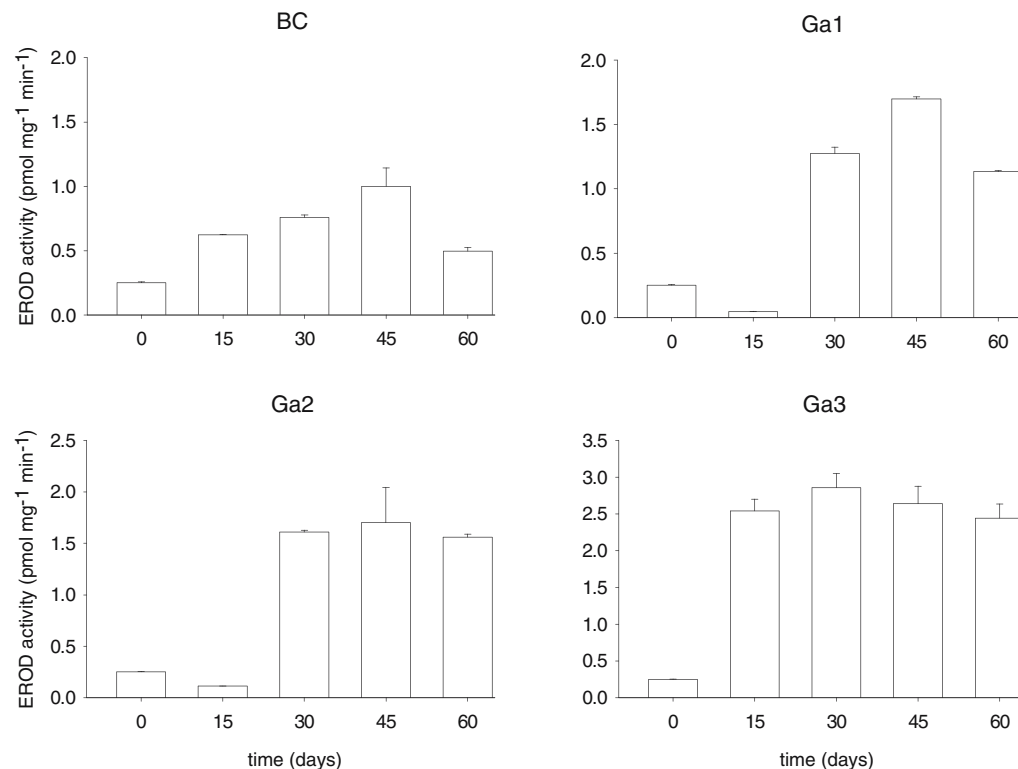
Contaminant		BC	Ga1	Ga2	Ga3
PAHs (mg kg <sup>-1</sup> )	TOC	1.07	0.60	1.19	2.00
	Fines (<63 µm)	1.04	0.06	0.03	0.01
	Total PAHs	ND	0.19	2.12	5.10
	Fluorene	ND	0.08	0.13	0.35
	Acenaphthene	ND	0.06	0.17	0.27
	Naphthalene	ND	0.31	0.63	1.40
	Phenanthrene	ND	0.10	0.15	1.36
	Anthracene	ND	0.02	0.03	0.18
	Fluoranthene	ND	0.12	0.18	0.10
	Pyrene	ND	0.09	0.13	0.39
	Benzo[a]anthracene	ND	0.05	0.09	0.20
	Chrysene	ND	0.08	0.12	0.39
	Benzo[fluoranthene	ND	0.11	0.18	0.06
	Benzo[e]pyrene	ND	0.08	0.13	0.16
	Benzo[a]pyrene	ND	0.05	0.09	0.10
	Perilene	ND	0.03	0.05	0.04
	Dibenzo[ah]anthracene	ND	0.01	0.02	0.02
	Indene[123-cd]pyrene	ND	0.02	0.02	0.02
	Benzo[ghi]perilene	ND	0.01	0.02	0.06
Metals (mg kg <sup>-1</sup> )	Cd	0.92	0.16	0.05	ND
	Cr	0.10	ND	2.00	1.51
	Cu	6.98	12.8	0.65	1.19
	Ni	0.06	1.71	0.42	0.66
	Pb	2.28	2.73	1.14	1.26
	Zn	21.3	14.7	3.95	6.45
	Hg	ND	ND	0.01	ND

Not detected is expressed by n.d. Table adapted from Morales-Caselles et al. (2006).

concentration of metals (Cd, Cr, Cu, Ni, Pb, Zn, Hg) and PAHs (Fluorene, Acenaphthene, Naphthalene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo[a]anthracene, Chrysene, Benzo[fluoranthene, Benzo[e]pyrene, Benzo[a]pyrene, Perilene, Dibenzo[ah]anthracene, Indene[123-cd]pyrene, Benzo[ghi]perilene) in the different sediment used in the test (Riba et al., 2004a). Sediments from the reference station (BC) show low values of metals while PAHs were not detected. The highest values of PAHs have been measured in sediments from the station Ga3 (5.10 mg kg<sup>-1</sup> dry weight) followed by the station Ga2 (2.12 mg kg<sup>-1</sup> dry weight) and Ga1 (0.19 mg kg<sup>-1</sup> dry weight). The concentration of metals in sediments from the stations located in Galicia is similar to those measured in the reference station (BC). Previous studies pointed out the possible amount of some metals concentration such as Ni, V, Cu, Pb and Zn (Albaigés & Bayona, 2003; CSIC, 2003; Prego & Cobelo-García, 2003, 2004) from the oil spill although they were not observed at high levels in our study.

Figure 2 shows the values of the EROD activity measured in liver samples of the *S. aurata* exposed to sediments treatments throughout 60 days. In general, EROD activity increases with the presence of PAHs in the sediment samples (Ga3>Ga2>Ga1>BC). Several studies agree that the use of EROD induction in fish is particularly well suited for detection of PAH exposure, because parent compounds may often not be detected in tissues (Whyte, Jung, Schmitt, & Tillitt, 2000).

The study of the behaviour of EROD activity during the exposure period for Ga3 shows that EROD activity increases significantly at the beginning of the experiments until day number 15 (2.4 pmol/mg/min of protein) and maximum levels are reached (2.9 pmol/mg/min of protein) on day 30. The measures of this biomarker in the liver of the organisms exposed to sediments from Ga1 and Ga2 show a lower increase than in the case of exposure to sediment in Ga3 and reach the maximum later than Ga3, the day 45 (about 1.7 pmol/mg/min of protein for both curves). In the course of the experiment the



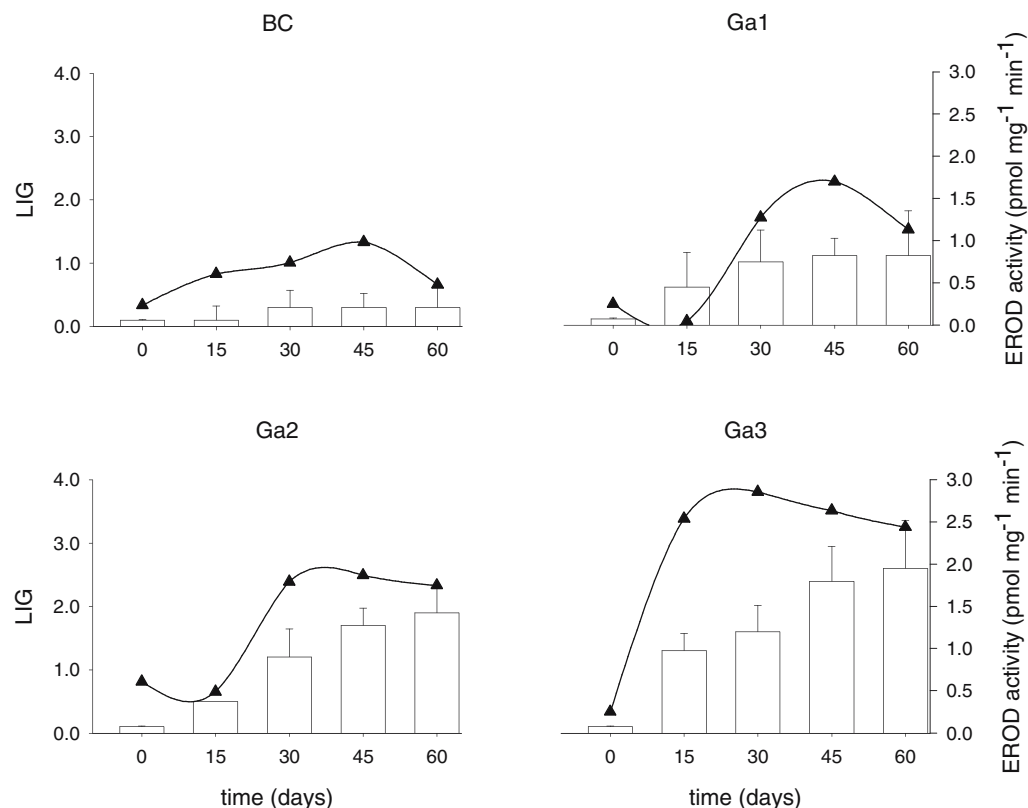
**Figure 2** EROD activity in picomoles per milligram per minute of protein in liver samples of *S. aurata* exposed to sediments from Galicia (Ga#) and control (BC) during the 60 days of bioassay.

EROD activity for Ga3 – which is the station with the greatest amount of PAHs in their sediments, 5.1 mg kg<sup>-1</sup> dry weight – is always higher than the EROD activity for Ga1 and Ga2. These sites (Ga1 and Ga2) show a similar behaviour along the time. For all the stations it is shown a slight decrease of the induction of this biomarker of exposure after the day 30 for Ga3 and after day 45 for the other three stations (including the reference station).

The histological alterations observed in target tissues (gill and liver) of fish exposed to sediment collected along the 60 days in the different stations were mainly in gills, which showed shortening of secondary lamellae, presence of edematous areas in distal portion of lamellae, hypertrophy and hyperplasia, necrosis and lost of cells epithelial in the organisms exposed to the Galician sediments; fusion of the secondary was detected specially in organisms

exposed to sediments from Ga3. Liver showed lesions such as vacuolization of hepatocytes, necrosis and decrease of the zymogen granules of the exocrine pancreas in the organisms exposed to the Galician sediments. In general, an increase of cytoplasmic basophilia was detected in the liver and exocrine pancreas of all exposed fish that seems related to the increase of PAHs.

In Figures 3 and 4 the summarized results of the histopathological alteration are shown as the index of lesions. The index for gills (LIG) increases with the presence of PAHs in the sediment samples (Ga3 > Ga2 > Ga1 > BC) and, in general, LIG increases along the time of exposure (Figure 3). The value of LIG is maximum the day 60 of the experiment and the highest frequency corresponds to the damages observed in the gills of the organisms exposed to sediment from Ga3 (LIG=2.6), followed by Ga2



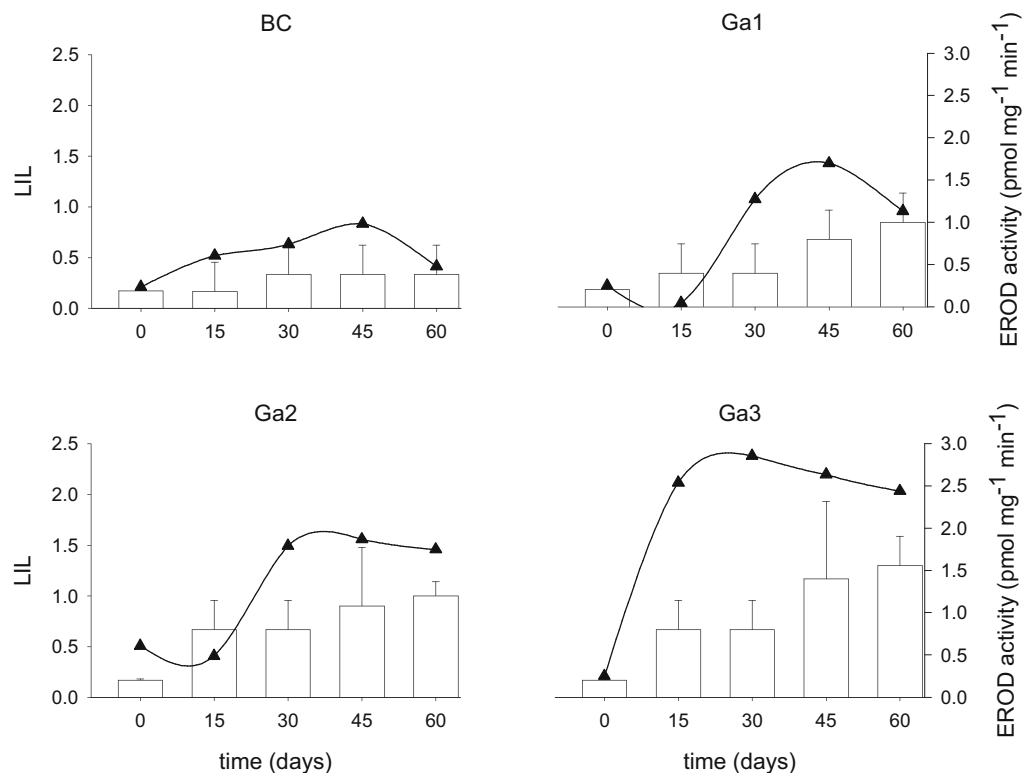
**Figure 3** The General Index of Lesions measured in the fish *Sparus aurata* for gills (LIG) along the period of exposure to the sediments are represented by bars. EROD activity in

picomoles per milligram per minute of protein in liver samples of *S. aurata* along the duration of the experiment for the control (BC) and Galician (Ga#) sites is represented by curves.

(LIG=1.9) and Ga1 (LIG=1.1). A similar behaviour can be observed for the index of lesions determined in liver (LIL), where Ga3 presents the highest value (Ga3: LIL=1.3; Ga2: LIL=1.1; Ga1: LIL=0.8; all of them evaluated at the end of the exposure period, after 60 days). The values of LIG were higher than LIL throughout the whole bioassay for all the stations. Gill is a multifunctional organ sensitive to chemicals in water, since gill filaments and lamellae provide a very large surface area for direct and continuous contact with contaminants in water.

The EROD activity is used as a biomarker of exposure to lipophilic organic contaminants and measures the enzymatic activity of the phase I catalyzed by the complex CYP1A; the complex CYP1A transforms some lipophilic xenobiotics in metabolites more water soluble, so that they are easier

to excrete. However, some of these new compounds are highly reactive and more toxic than the original contaminant, and they might interact with biological macromolecules (Parkinson, 1995) producing lesions. In the fishes exposed to sediments from the station Ga3, it seems that there is a first phase where EROD activity is induced (days 0–30) while there are some histopathological damages. When the activity reaches a maximum and begins to decrease (days 30–60), the histopathological alterations continue increasing and the frequency of the lesions is higher. This mechanism of induction of histopathological damages when hepatic EROD decreases can be shown in the three stations affected by the *Prestige* oil spill, although it is produced faster and with higher intensity and frequency in the organisms exposed to sediments from Ga3, which also shows the highest PAHs concentra-



**Figure 4** The General Index of Lesions measured in the fish *Sparus aurata* for liver (LIL) along the period of exposure to the sediments are represented by bars. EROD activity in

picomoles per milligram per minute of protein in liver samples of *S. aurata* along the duration of the experiment for the control (BC) and Galician (Ga#) sites is represented by curves.

tion in sediment. This behaviour could be related to the production of toxic metabolites as secondary products in the detoxification process where the EROD activates. It seems that when EROD activity stabilizes or disappears from the cells, the tissues get more defenceless to organic compounds (in this case PAHs), and histopathological damages are caused with more intensity and frequency.

#### 4 Conclusions

This study shows that the comparison between chemical analysis and the different toxic responses (biomarkers of exposure and effect) is a useful tool to determine the quality of the studied sediments that were affected by the oil spill. The results obtained demonstrate that PAHs analyzed in sediments from

Galicia were the chemicals responsible for the measured adverse effects (biomarkers of exposure and effect). The toxicokinetic approach used in this study proposes a mechanism that can explain the histopathological damage associated with the exposure of fish to environmental samples contaminated by PAHs from an oil spill (Prestige, 2002). It gives us the possibility to compare entire curves of behaviour instead of numerical data (endpoint). Besides, it permits to understand better the kinetic of the toxicity based on the role of a detoxification system such as the CYP1A complex. It has been proved the importance of the use of chronic bioassays which provide long-term information of the effects of the exposure to a toxic compound analyzed in environmental samples.

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## **Role of biomarkers to assess oil-contaminated sediment quality using toxicity tests with clams and crabs**

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### **Abstract**

A 28-day bioassay was conducted with two invertebrate species with different feeding habits, the clam *Ruditapes philippinarum* and the shore crab *Carcinus maenas*. The purpose of the study was to assess the quality of sediments affected by oil spills in different areas of the Spanish Coast. The organisms were exposed to environmental samples of oil-contaminated sediments during four weeks, and after the experiment a suite of biomarkers of exposure was measured: the phase I detoxification system was assessed by Ethoxyresorufin O-deethylase (EROD) activity; glutathione-S-transferase (GST) is a phase II detoxification enzyme but is also implicated in oxidative stress events; glutathione peroxidase (GPX), glutathione reductase (GR) and the ferric reducing ability of plasma (FRAP) assay were analyzed to determine the antioxidant activity of the tissues. The biomarker results were correlated with the chemical compounds bound to sediments (PAHs, PCBs, Zn, Cd, Pb, Cu, Ni, Co, V) and a principal component analysis was carried out with the purpose of linking all the variables, and to detect those contaminated sediments potentially harmful to the biota. Results showed induction of biomarkers in both invertebrate species and significant differences ( $p < 0.05$ ;  $p < 0.01$ ) were

established among sediments affected by different spills. The use of the selected biomarkers together with the sediment chemical analysis assesses the bioavailability of contaminants and has proven to be a suitable tool to monitor the environmental quality of sediments affected by oil spills.

*Keywords: PAHs, toxicity, bioassay, oil spill, WOE*

## **1. Introduction**

The presence of persistent pollutants related to oil spills such as PAHs and PCBs and toxic metals (Cd, Pb, Zn, Cu, Ni, Co, V, etc) in different compartments of the marine environment has become a major threat to the health of marine ecosystems due to accumulation of their residues in the tissues of marine organisms [1]. Biomarkers have been shown to be useful tools in characterizing the health status of animals from affected areas, where complex mixtures of pollutants are usually present [2, 3, 4]. Biomarkers present the inherent capacity to detect early biological effects within the organism and to monitor the temporal progression (or regression) of the disturbance of various levels of biological organization [5]. Under controlled conditions in the laboratory, it is relatively straightforward to standardise biomarker assays and to regulate the chemical exposures that organisms receive, so that cause-effect and indeed, exposure-relationships, can be established [6].

The fluctuation of different biomarkers in response to different toxicants provides a pattern of results which can give clues as to the type of pollutant that is causing the observed effect [6]. Biomarkers have been previously used to assess oil spill episodes [5, 7, 8, 9]. In the present study a suite of biomarkers was chosen in order to investigate biological responses of organisms exposed to oil-contaminated sediments from the Galician Coast (NW Spain), acutely affected by a fuel spill (Prestige, 2002), and the Bay of Algeciras (S Spain), chronically affected by different spills. Ethoxyresorufin O-deethylase (EROD)

was selected as the phase I detoxification enzyme implicated in monooxygenation reactions of dioxins and PAHs. Glutathione-S-transferase (GST) is a phase II detoxification enzyme but is also implicated in oxidative stress events, while glutathione peroxidase (GPX) and glutathione reductase (GR) were chosen as antioxidant enzymes together with the ferric reducing ability of plasma (FRAP) assay. The combination of biological responses and chemical data of the sediment helps identify the integrated impact of chemical contamination on organisms. Many authors agree that sediment quality is best determined by integrating the information obtained from measures of chemical concentration and from specific tests to determine sediment toxicity [10, 11].

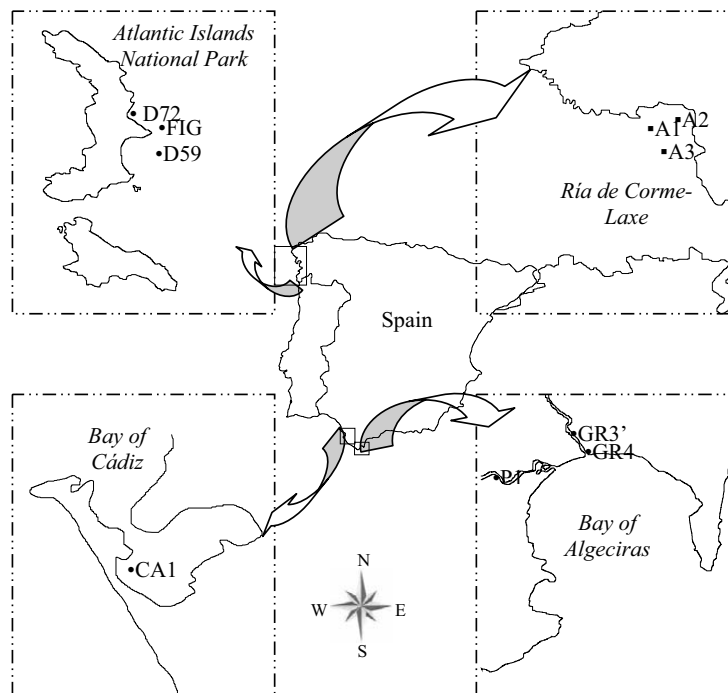
The purpose of this work is to test the suitability of using a set of biomarkers in two invertebrate species, the clam *Ruditapes philippinarum* and the crab *Carcinus maenas*, in order to assess the environmental quality of sediments affected by oil spills. To achieve this objective the selected biomarkers were linked with the concentration of contaminants in the sediments and the results are discussed.

## **2. Material and methods**

### **2.1. Approach**

The sediments employed in the present study were collected in two areas of the Spanish coast affected by oil spills. The area of the Galician coast (NW Spain) suffered the acute impact of the *Prestige* oil spill in 2002 whereas the Bay of Algeciras (S Spain) is continuously affected by minor spills, including oil and other contaminants from industries and discharges from commercial shipping activities [12]. A reference site with no organic pollution was selected in the Bay

of Cádiz (S Spain). This site has been widely validated as a reference area [9, 12, 13, 14]. The 10 selected study sites are shown in Figure 1.



**Figure 1.** Map of the coastal area of Galicia showing the locations of the sampling stations. FIG, D59 and D72 refers to the stations located in the Cies Island in the Atlantic Island National Park and A1, A2 and A3 to those in the Bay of Corme-Laxe. The stations located in the Bay of Algeciras are GR3', GR4 and P1. The station CA located in the Bay of Cadiz corresponds to the sediment used as reference.

## 2.2. Bioassays

The clam *Ruditapes philippinarum* and the crab *Carcinus maenas* were obtained from an aquaculture farm and were kept under laboratory conditions

in tanks with continuous water replacement during 10 days for acclimation. 25-L tanks were employed to perform the bioassay with crabs, whereas 11-L aquariums were selected to carry out the experiment with clams. Sediment collected in the study sites was placed in replicate in the tanks: 4 L of sediment was put in the 25-L glass tanks and 2 L of sediment sample was placed in the 11-L aquariums. Clean sea water was then added and after particle settling, aeration was provided to maintain adequate oxygen concentrations (greater than 80% saturation). Subsequently, the organisms were transferred to the tanks, the laboratory conditions were controlled, the temperature was kept at  $19\pm1^{\circ}\text{C}$  and the natural photoperiod was maintained. The bioassays were performed in duplicate and lasted 28 days; over this time the water in the tanks was replaced and the crabs were fed every week with a mixed diet of mussels or fish, while the clams were fed with an algae preparation.

### 2.3. Biochemical analysis

After 28 days of the exposure period a survey was carried out and the hepatopancreas (in crabs) and digestive gland (in clams) were extracted and kept at  $-80^{\circ}\text{C}$  prior to homogenization. The samples were homogenized according to the procedure developed by Lafontaine et al. [15].

Following homogenization, the samples were centrifuged at 10,000g for 30 min, and the supernatant was used for the biomarker determination. Mixed function oxygenase activity, which is the first mode of detoxification of many organic pollutants, was measured using the adapted EROD assay [16]. The FRAP assay allows a measure of the antioxidant capacity and was carried out as described by Benzie and Strain [17]. The antioxidant Glutathione-S-transferase (GST) activity was determined by monitoring the rate of conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm [18]. The

oxidation of 1 mM NADPH by Glutathione Reductase activity (GR) in the presence of 10 mM oxidized glutathione was also monitored at 340 nm [18]. The phase II metabolizing enzyme Glutathione Peroxidase activity (GPX) was measured according to McFarland et al. [18]. All the biomarker responses were normalized to the total protein content [19].

#### 2.4. Chemical analysis

The analyses of PAHs and PCBs were carried out according to USEPA SW-846 Method 827C78082. Briefly, following recommendations by Riba et al. [20], dried samples were Soxhlet extracted with n-hexane for 18 h, and the extracts were isolated by column chromatography on Florisile alumino-silica. The PCBs and PAHs were eluted and their fractions were dried in a rotating evaporator and re-dissolved in isooctane. The aromatic fractions were analyzed using a Hewlett Packard (HP) 5890 Series II gas chromatographer coupled with an HP 5970 mass spectrometer. The PAHs were analyzed by GC-MS using selected ion monitoring (SIM). Analysis of PCBs such as AROCLOR 1242 and AROCLOR 1260 was performed using the same instrument with an electron capture detector (GC/ECD). For both sets of organic chemicals, PAHs and AROCLOR, the analytical procedure showed agreement with the certified values of more than 90%.

A trace metal analysis was carried out as described by Casado-Martínez et al. [21]. Briefly, 2.5 g of sediments (<0.065 mm) were placed in Teflon containers and were digested in a microwave oven (400W, 15 min, twice) with 2N HNO<sub>3</sub>. The extracts were purified by passing them through a C-18 column and metal analyses were performed by anodic voltamperometry (-Zn, Cd, Pb, Ni, Co and Cu- Metrohm Application Bulletin N° 147; - V- Metrohm Application Note N° V-81). The cold vapour technique was used for Hg and

was quantified using atomic absorption spectrometry. The analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) and showed a recovery greater than 90% of the certified concentration.

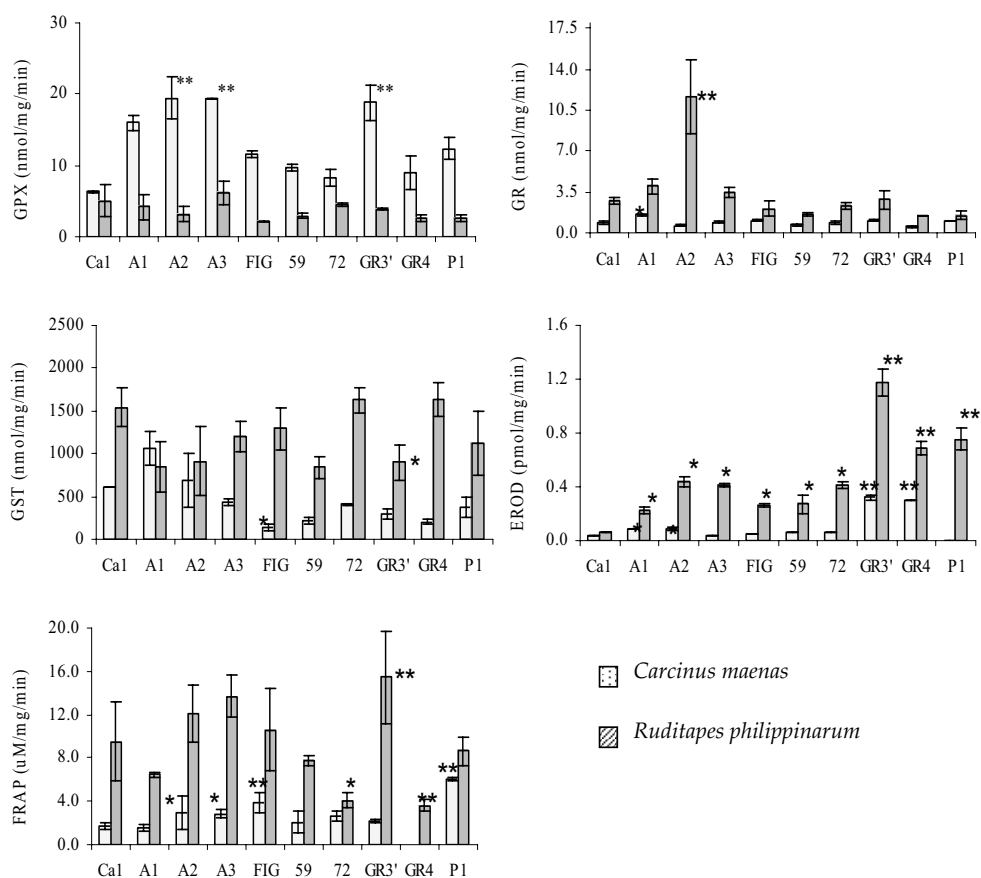
## 2.5. Statistical analysis

The biomarker results were analyzed with the ANOVA and Tukey test with the aim of determining significant differences ( $p < 0.05$ ;  $p < 0.01$ ) between the results obtained for the reference site and the other sampling sites (SPSS 11.5). The chemical concentrations in sediments and biomarker responses were correlated with the Pearson analysis ( $p < 0.05$ ) in order to detect relationships between the variables (STATISTICA 6.0). Finally a multivariate analysis was carried out with the purpose of linking chemical and biological data; the principal component analysis (PCA) was used as the extraction procedure to derive a reduced number of new variables (factors) as linear combinations of the original variables (STATISTICA 6.0) [22].

# 3. Results

## 3.1. Biomarker responses

The biomarker responses in crabs and clams after 28 days of exposure to the sediment samples are shown in Figure 2. The GPX activity results (Figure 2.a) showed the lowest values in *C. maenas* exposed to the reference sediment. Significant differences ( $p < 0.01$ ) in GPX induction were detected between crabs exposed to the reference sediment and crabs exposed to test sediments collected in A2 and A3 in Corme-Laxe and GR3' in the Bay of Algeciras. The induction of this biomarker in clams did not present significant differences between treatments. The induction of the antioxidant biomarker GR (Figure 2.b) in crabs exposed to sediment from A1 and clams from the A2 treatment was



**Figure 2.** General health biomarkers for both invertebrate species, the clam *Ruditapes philippinarum* and the crab *Carcinus maenas*: glutathione peroxidase activity GPX (nmol/min/mg prot), glutathione transferase GST activity (nmol/min/mg prot), glutathione reductase GR activity (nmol/min/mg prot), ferric reducing ability of plasma FRAP activity (μM/mg/min) and EROD activity (pmol/mg/min). Asterisks indicate significant differences with the reference treatment CA1 (\*p < 0.05; \*\*p < 0.01).

significantly different ( $p < 0.05$  and  $p < 0.01$  respectively) from the reference site; on the other hand crabs exposed to sediment from FIG and clams exposed to GR3' presented significant differences ( $p < 0.05$ ) and lower values than the reference site CA. In relation to the phase I detoxification system, clams from all treatments showed significant differences with the reference site in EROD activity (Figure 2.d); these differences were greater ( $p < 0.01$ ) for those clams that had been exposed to the sediments collected in the Bay of Algeciras (GR3', GR4 and P1). EROD induction in crabs from A1, A2 in Corme-Laxe, GR3' and GR4 in the Bay of Algeciras was also significantly different from the reference station ( $p < 0.05$ ). The antioxidant activity obtained from the FRAP assay (Figure 2.e) showed significant differences between crabs exposed to A2 ( $p < 0.05$ ), A3 ( $p < 0.05$ ), FIG ( $p < 0.05$ ), P1 ( $p < 0.01$ ) and the reference site; in the case of clams, GR3' ( $p < 0.01$ ), GR4 ( $p < 0.01$ ) and D72 ( $p < 0.05$ ) presented significant differences to the reference site, although GR3' showed higher values than CA whereas GR4 and D72 presented lower values.

### 3.2. Chemical analysis

Results of the chemical analysis are shown in Table1. Sediments from the reference site did not present organic contamination whereas sediments from the Bay of Algeciras (GR3' > GR4 > P1), chronically affected by different spill and Corme-Laxe (A1 > A2 > A3) presented higher concentrations of PAHs in their sediments than sites from the Cies Island (59 > FIG > 72). In general, chemical analysis does not present a prevailing tendency in the concentration of metals among sediments from the different areas. Samples collected in the site GR3 from the Bay of Algeciras presented the highest values of Ni ( $74.7 \text{ mg Kg}^{-1}$ ), whereas Zn, and Pb presents their maximums in sediments from the area of Corme-Laxe (A1 and A3).

**Table 1.** Concentration of PAHs and PCBs ( $\mu\text{g kg}^{-1}$  dry weight) and metals ( $\text{mg kg}^{-1}$  dry weight) in the sediment samples used in the bioassays.

	Reference	Corme-Laxe			Cíes Island			Bay of Algeciras		
	Ca1	A1	A2	A3	FIG	59	72	GR3'	GR4	P1
<b>PAHs</b>	n.d.	820	558	537	257	370	239	2961	802	641
<b>PCBs</b>	n.d.	2.28	4.29	2.60	n.d.	6.52	4.76	22.0	1.75	0.84
<b>Zn</b>	21.3	244	31.8	65.7	76.2	43.4	37.5	138	35.3	56.7
<b>Cd</b>	0.92	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.17	0.10	0.12
<b>Pb</b>	2.28	14.3	4.25	44.0	26.6	9.13	6.54	21.6	6.21	12.3
<b>Cu</b>	6.98	19.1	n.d.	22.1	18.9	n.d.	31.6	5.01	3.67	75.2
<b>Ni</b>	0.06	7.03	5.61	9.39	12.0	6.88	5.02	74.7	13.1	13.3
<b>Co</b>	3.40	0.67	0.37	1.21	0.52	n.d.	0.87	12.8	5.59	n.d.
<b>V</b>	80.0	5.94	2.34	13.4	n.d.	n.d.	n.d.	26.1	n.d.	6.84

### 3.3. Correlation between variables

A correlation analysis was conducted in order to detect relationships between the presence of contaminants in the sediments and the induction of biomarkers in the invertebrates exposed to the same as biological mechanisms to defend against these compounds. Significant correlations ( $p < 0.05$  and  $p < 0.01$ ) were observed between sediment contamination and biological responses in the organisms exposed (table 2). Similarly, significant associations ( $p < 0.05$  and  $p < 0.01$ ) were observed between organic contaminants (PAHs and PCBs) bound to sediments, the metals Ni and Co and the induction of EROD activity in both clams and crabs after the 28-day exposure time.

**Table 2.** Pearson correlation (\*p<0.05, \*\*p<0.01) results among chemical compounds bound to sediments and biomarkers: Ethoxymesorufin O-deethylase (EROD) activity, glutathione-S-transferase (GST) activity, glutathione peroxidase (GPX) activity, glutathione reductase (GR) activity and ferric reducing ability of plasma (FRAP) activity.

	PAHs	PCBs	Zn	Cd	Pb	Cu	Ni	Co	V	GPX	GPX	GR	GST	GST	EROD	EROD	FRAP
										crab	clam	crab	clam	crab	clam	crab	clam
PAHs																	
PCBs	.905**																
Zn		.905**															
Cd									.970**								
Pb																	
Cu																	
Ni																	
Co																	
V																	
GPX-crab																	
GPX-clam																	
GR-crab																	
GR-clam																	
GST-crab																	
GST-clam																	
EROD-crab																	
EROD-clam																	
FRAP-crab																	
FRAP-clam																	

Meanwhile, the antioxidant GR and FRAP activity measured in both species was significantly ( $p < 0.01$ ) correlated with the presence of Zn and Cu in the sediment respectively. A relationship has been shown between the metals V and Cd ( $p < 0.01$ ) although this association did not present any relationship with the biomarkers studied.

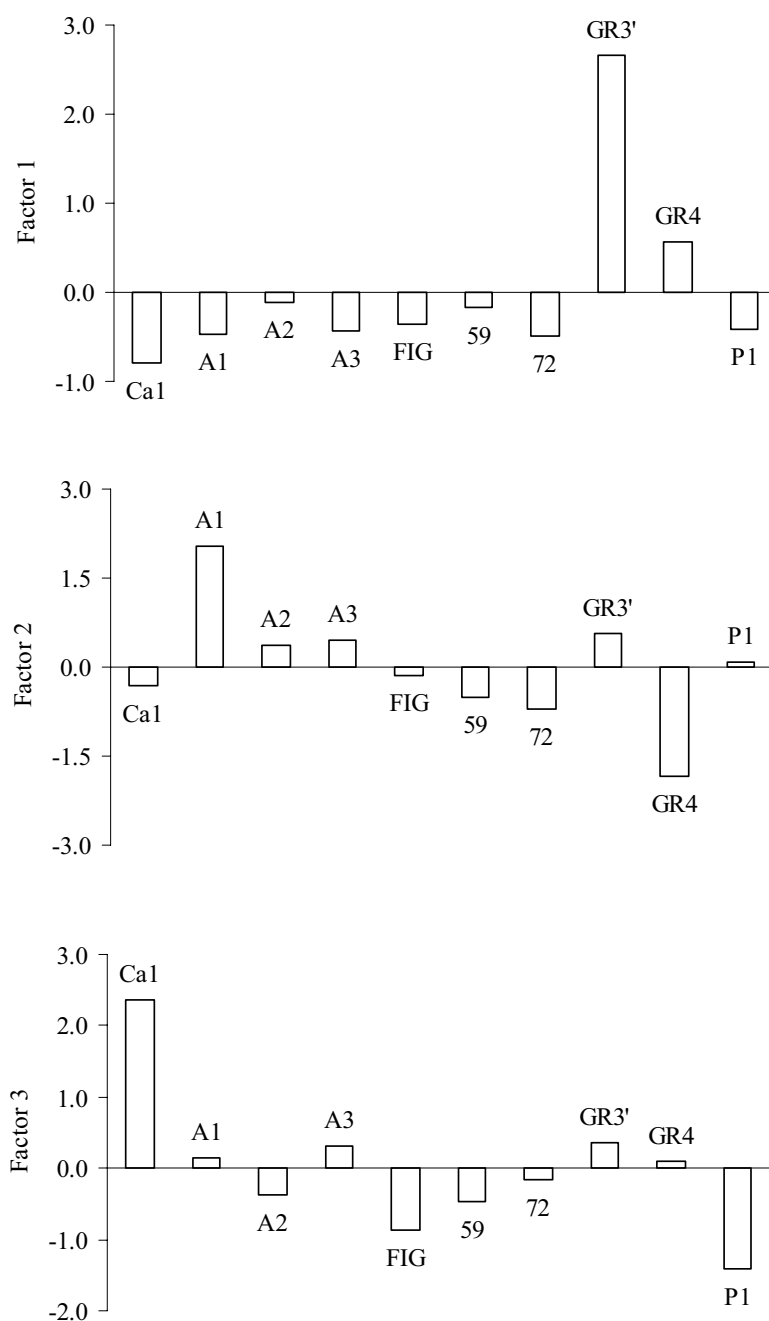
### 3.4. Principal components analysis

Based on correlations between the chemical and biological results, the principal components analysis enables grouping of the 19 original variables into 4 new factors which account for 63.3 % of the variance. The purpose of this analysis is to reduce the number of variables with the minimum loss of information in order to simplify the interpretation of the results. In the present study, it was decided to interpret a group of variables as being associated with a particular component where the loading was 0.30 or higher (Table 3), approximating to Comreys' cut-off of 0.55 [23] for a good association between an original variable and a factor. Factor 1 (31.2 %) links the concentration of PAHs, PCBs, Ni and Co in sediments with the induction of EROD activity in clams and crabs, the GPX activity induction in crabs and the FRAP activity in clams after 28 days of exposure. The metals Zn and Pb in sediment are related with GPX and GR activities in both crabs and clams, GST activity in crabs and FRAP induction in clams as defined by Factor 2 (17.3 %). Factor 3 (14.9 %), with negative loading, groups Cu with the induction of GPX and FRAP activities in crabs after 28 days of exposure to the sediments.

After defining the meaning of each factor, the PCA enables identification of the importance of each factor at each study site by using the factor score. Figure 3 shows the Factor score at each of the stations. The influence of Factor 1, related with the biomarker response of the organic contaminants (PAHs and PCHs), and the metals Co and Ni in sediments, is prevalent in the stations GR3' (2.66) and GR4 (0.57) from the Bay of Algeciras; Factor 2, which explains the

**Table 3.** Sorted rotated factor loadings of 19 variables for the three principal factors resulting from the multivariate analysis of results obtained from the chemical analysis and the biomarker responses in crabs and clams: Ethoxyresorufin O-deethylase (EROD) activity, glutathione-S-transferase (GST) activity, glutathione peroxidase (GPX) activity, glutathione reductase (GR) activity and ferric reducing ability of plasma (FRAP) activity.

	<b>FACTOR 1</b>	<b>FACTOR 2</b>	<b>FACTOR 3</b>
	31.23%	17.25%	14.85%
<b>PAHs</b>	0.95	—	—
<b>PCBs</b>	0.91	—	—
<b>Zn</b>	—	0.80	—
<b>Cd</b>	—	—	—
<b>Pb</b>	—	0.34	—
<b>Cu</b>	—	—	-0.49
<b>Ni</b>	0.95	—	—
<b>Co</b>	0.91	—	—
<b>V</b>	—	—	—
<b>GPX-crab-28</b>	0.48	0.38	-0.30
<b>GPX-clam-28</b>	—	0.31	—
<b>GR-crab-28</b>	—	0.82	—
<b>GR-clam-28</b>	—	0.34	—
<b>GST-crab-28</b>	—	0.73	—
<b>GST-clam-28</b>	—	—	—
<b>EROD-crab-28</b>	0.86	—	—
<b>EROD-clam-28</b>	0.85	—	—
<b>FRAP-crab-28</b>	—	—	-0.59
<b>FRAP-clam-28</b>	0.42	0.44	—



**Figure 3.** Factor loadings for the three principal factors resulting from the multivariate analysis of results obtained from the chemical analysis and the suite of biomarkers.

relationship between the content of Zn and Pb in the sediment and the biomarker responses in clams and crabs, presents a positive loading in the stations A1 (2.02), A2 (0.36) and A3 (0.46) from Corme-Laxe and GR3` (0.58) and P1 (0.07) from the Bay of Algeciras. Finally, Factor 3, which with negative loading relates Cu with some biomarker responses (GPX and FRAP induction in crabs) shows a prevalence (negative scores) in the stations from the Cies Islands FIG (-0.88), 59 (-0.46), 72 (-0.16), and A2 (-0.38) from Corme-Laxe and P1 (-1.40) from the Bay of Algeciras.

#### **4. Discussion**

The present study analyses the relationship between biomarker responses in organisms exposed to sediments contaminated by oil spills in NW and S Spain and their chemical content. Two invertebrate species with different feeding habits have been employed in this research as bioindicator species, the shore crab *Carcinus maenas* and the clam *Ruditapes Philippinarum*.

Despite the difficulty of testing complex mixtures of contaminants, the results have shown clear relationships between the different antioxidant enzymes in the tested organisms in the presence of metals. The Phase I detoxification system measured by the EROD activity was related to organic contaminants (PAHs and PCBs) and metals bound to complex organic mixtures (Ni and Co). These contaminants, especially PAHs and Ni, have often been linked to oil spills. Antioxidant response (GPX in crabs and FRAP in clams) was also identified for these compounds. EROD activity is often used as a biomarker of exposure to lipophilic organic contaminants and measures the enzymatic activity of phase I catalyzed by the complex CYP1A. The complex CYP1A transforms some lipophilic xenobiotics into more water-soluble metabolites, so that they are easier to excrete. Induction of EROD activity has been previously

reported in crabs and clams subsequent to organic pollutant exposure [9, 15, 24]. The highest values of EROD activity were obtained in organisms exposed to sediments from the Bay of Algeciras, and the multivariate analysis linked this induction with organic compounds (PAHs and PCBs). In addition, the induction of antioxidant enzymes measured by the GPX and FRAP analysis was linked with organic contaminants in sediments by Factor 1; Cheung et al. [25] demonstrated that some PAHs are potent oxidative stress inducers in the marine mussel, *Perna viridis*, and obtained increasing values of antioxidant parameters, including GPX. Many pollutants (or their metabolites) may elicit toxicity related to oxidative stress. Oxygen toxicity can be a potent oxidant capable of reacting with critical cellular macromolecules, possibly leading to DNA damage and cell death. Defense systems that tend to inhibit oxyradical formation include antioxidant enzymes such as glutathione reductase (GR) and glutathione peroxidase (GPX) [9]. It is well known that GPX transforms organohydroperoxide to alcohol and water at the expense of GSH [26]. Thus, GPX activity is likely to be influenced by the GSH level and GR activity, which regulates the level of GSH [25]. The results obtained in the multivariate analysis demonstrated a relationship of the antioxidant biomarkers GPX and GR in both invertebrate species due to the presence of Zn and Pb in sediments. This means that these metals are producing some stress in the exposed organisms, as is reflected in the antioxidant responses.

The induction of lipid peroxidation by copper is well-known in other invertebrates [27], which could explain the connection between Cu and the induction of antioxidant enzymes explained by Factor 3 in the MAA. It is important to note that Cu belongs to a group of metals that are redox-active and are capable of directly generating free radicals [28] which may lead to antioxidant defense responses. Previous studies also reported an induction of

GPX activity in *Mytilus galloprovincialis* exposed to copper in controlled conditions or to complex mixtures of metals in field conditions [29]. The induction of FRAP activity in crabs and clams seems to be less relevant than other biomarkers related to oxidative stress, although the correlations observed, especially with metals, suggest the importance of analyzing a group of biomarkers rather than single use.

GST activity induced in crabs has been related to Zn and Pb contamination in the multivariate analysis of Factor 2. Glutathione transferases phase II detoxification enzymes which utilize glutathione (GSH) as a substrate in reactions which permit the biotransformation and disposal of exogenous compounds [30]; the induction of GST activity in *Carcinus maenas* has been previously related with metal contamination [9].

The results obtained in the present study showed the activation of different defence systems in both the organisms tested. This was mainly related with an input of chronic fuel oil contamination into the studied sediments, predominantly in the Bay of Algeciras, followed by Corme-Laxe, which was affected by an acute oil impact; these results correspond with histopathological damage observed in the tissue of crabs and clams under laboratory conditions exposed to these sediments (personal observations). The Ni and Co sediment content correlates with the organic contaminants which are usual in the case of hydrocarbon contamination episodes. Zn and Pb metal contamination was also detected in the area of Corme-Laxe and the Bay of Algeciras, producing stress in the animals exposed; besides, a source of Cu in Cíes and Algeciras was linked with antioxidant responses in the organisms tested. The presence of metals in these areas suggests that other alternative sources of contaminants should be investigated apart from the *Prestige* oil spill.

Both species employed in the present study, the clam *Ruditapes philippinarum* and the crab *Carcinus maenas*, have biochemically responded to the contamination present in the sediments. All the biomarkers presented higher values in the digestive gland of clams than in the hepatopancreas of crabs, except for GST. The use of two invertebrate species with different feeding habits allows a more complete study of the biological effects of contaminants bound to sediments. According to other authors [31, 32], the information provided by each biomarker individually is of limited relevance, as there is a considerable likelihood of misinterpretation; thus, biomarkers are best used as selected batteries of tests rather than individually. In addition, combining chemical analysis with suites of biomarkers addresses the need for more pragmatic environmental assessment techniques linking environmental degradation with its causes [2]. The higher sensitivity of sublethal bioassays compared to acute toxicity tests indicates the advantages of incorporating this approach as part of a more complete and integrated study based on a weight-of-evidence approach, as previously recommended by some authors [8, 9, 33].

## 5. Conclusions

In the present study, an evaluation has been carried out of the environmental quality of coastal areas affected by different contaminants. The biomarkers have demonstrated that they are activated depending on the kind and level of contamination and have proven to be a suitable tool to assess oil-contaminated sediments. The clam *Ruditapes philippinarum* and the crab *Carcinus maenas* have demonstrated the importance of using different species of organisms when assessing environmental management and have responded satisfactorily to the contamination present in the sediments, demonstrating the bioavailability of organic and inorganic contaminants related to oil spills. The

application of the methodology in two areas affected in different manners by oil spills (acutely: Galician coast and chronically: Bay of Algeciras) has shown how biomarkers are activated in diverse ways depending on the source of the pollutants.

This study has demonstrated the important role that biomarkers play as part of the Weight of Evidence Approach (WOE) and its use in the assessment of oil spills is strongly recommended.

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# Vitellogenin variation in the crab *Carcinus maenas* exposed to sediments affected by oil spills (Spain)

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## Abstract

In the present study the induction of vitellogenin has been studied as a biomarker of exposure in crabs in order to assess its relationship with contaminants bound to sediments [Zn, Pb, Cu, Ni, Co, V and polycyclic aromatic hydrocarbons (PAHs)] affected by different oil spills in Spain. Two different 28-days bioassays have been carried out both under field and laboratory conditions by exposing the crab *Carcinus maenas* to contaminated sediment samples. For the field approach the organisms were labelled and kept in cages located in the study sites during the exposure period. In the experiment conducted under laboratory conditions sediment from the stations was collected and carried to the laboratory where labelled crabs were placed in 20 L tanks with the sediment samples. For both bioassays haemolymph was extracted from the individuals the day 0 and 28 of exposure to determine the variation in the levels of vitellogenin after the bioassay. The Spanish sediments selected for this study had been affected in a different way by oil spills; the Galician Coast (NW Spain) was acutely impacted by the accident of the tanker *Prestige* (2002) whereas the Bay of Algeciras (South Spain) suffers chronically from continuous input of different contaminants from ships and industries located in the area, including oil spills. Results show a relationship between

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vitellogenin induction and contaminants. The variation of vitellogenin concentration was related to the presence of PAHs and the metals Pb, Ni and Cu in the sediment, which occurred mainly in the treatments from the Bay of Algeciras. In this sense, the study shows a partial recovery in the sediment quality in the Galician Coast three years after the spill, whereas the Bay of Algeciras is significantly more polluted than the sediments studied in the area of Galicia.

*Keywords: vitellogenin, oil spill, ecotoxicity, invertebrate, PAHs*

## **1. Introduction**

Sediments, as an important part of the ecosystem, are often studied to assess environmental quality. Complex mixtures of contaminants however can be examined with difficulty, thus the study of both chemical and toxicological effects turns out to be a suitable tool to achieve the objectives proposed in sediment quality assessment. Biomarkers can be defined as measurements of body fluids, cells or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response to such contaminants <sup>[1]</sup>. Combining chemical analysis with suites of biomarkers addresses the need for more pragmatic environmental assessment techniques linking environmental degradation with its causes <sup>[2]</sup>.

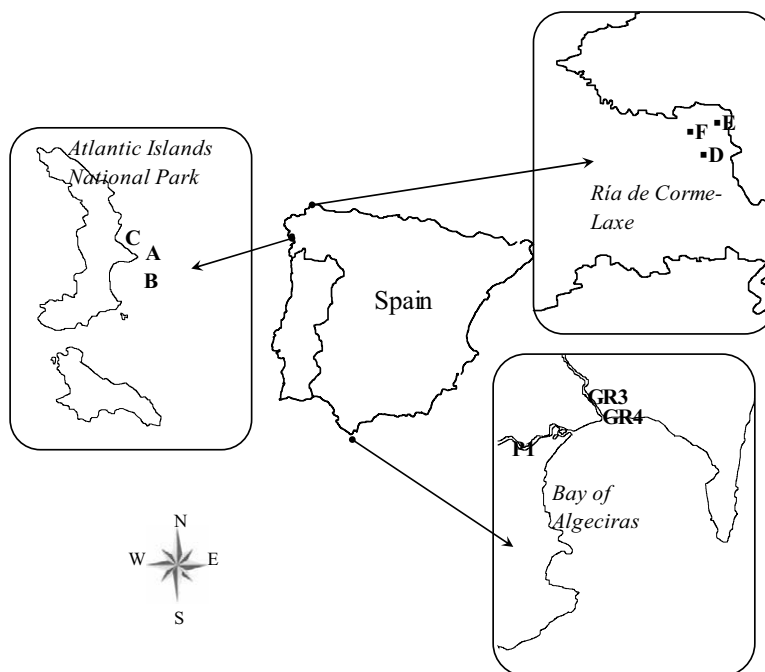
The chemicals introduced into the environment have the potential to interact with neuro-endocrine signaling cascades, resulting in signal perturbations <sup>[3]</sup>. Such altered signaling can result in modifications to development, maturation, reproduction, and other neuro-endocrine-regulated processes that hinder population sustainability <sup>[4]</sup>. Laboratory studies have demonstrated the susceptibility of crustaceans to toxicants and field studies have revealed evidence of endocrine disruption among various crustacean populations <sup>[5]</sup>.

Due to their biological and ecological characteristics crabs are suitable organisms for use in ecotoxicological studies <sup>[6]</sup>. In the present study vitellogenin (VTG) is used as a biomarker to assess the toxicity caused by oil spills on the crab *Carcinus maenas*. Vitellogenesis is a process by which female crustaceans produce and sequester nutrients into developing oocytes <sup>[7]</sup>. Vitellogenin is a protein produced in the hepatopancreas and transported through the haemolymph to the ovary, where it enters into growing oocytes <sup>[7]</sup>. The inhibition or stimulation of vitellogenin levels in haemolymph could provide a useful indicator of direct repercussions on the reproductive capacity in the female crabs <sup>[8]</sup>.

The aim of this study was to investigate the relationship between contaminated sediments with the induction of vitellogenin in the crab *Carcinus maenas* by exposing the organisms to sediments from the Galician Coast (NW Spain), three years after an oil spill (*Prestige*), and sediments from the Bay of Algeciras (S Spain), chronically impacted by spills of different contaminants including oil spills. Exposures were performed under field and laboratory conditions with the purpose of study the similarities and differences of both methodologies.

## **2. Material and methods**

The sediments selected for this study were affected in a different way by oil spills; the Galician Coast (NW Spain) was acutely impacted by the accident of the tanker *Prestige* (2002) whereas the Bay of Algeciras (S Spain) chronically suffers continuous input of different contaminants coming from ships and industries located in the area, including oil spills. Figure 1 shows the location of the study sites.



**Figure 1.** Map of study sites: the coastal area of Galicia showing the locations of the sampling stations. A, B and C refers to the stations located in the Cies Island in the Atlantic Island National Park and D, E and F to those in the Bay of Corme-Laxe. The stations located in the Bay of Algeciras are GR3, GR4 and P1.

## 2.1. Sediment sampling and characterization

Sediments from the selected sites were carried to the laboratory and were sampled for physical characterization and chemical quantification. The analyses of PAHs were carried out according to USEPA SW-846 Method 827C78082 <sup>[9]</sup>. For trace metal analyses (Zn, Pb, Cu, Ni, Co, V), the sediments were digested as described by Loring and Rantala <sup>[10]</sup> and then measured by atomic absorption spectrophotometry (AAS). Organic carbon content was determined using the

method of Gaudette et al. <sup>[11]</sup> with the El Rayis <sup>[12]</sup> modification. For sediment grain size, an aliquot of wet sediment was analyzed using a Frisch laser particle sizer (model Analysette 22) following the method reported by DelValls and Chapman <sup>[13]</sup>.

## 2.2. Toxicity tests

Intermoult females crabs were collected from a clean site in the Gulf of Cádiz and were acclimatized for two weeks in the laboratory. After that period the sediment samples were placed in 20-L aquariums and sea water was added (1:4). Aeration was provided after the sediment had settled down. Crabs were labelled and a number of them were placed in the aquariums in the laboratory (8 per aquarium) and in the cages which were transferred to the study sites. The bioassays run in replicate and lasted 28 days.

## 2.3. Vitellogenin determination

Haemolymph samples were taken from the base of a walking leg using a syringe the days 0 and 28 of the bioassay. The samples were transferred to microcentrifuge tubes and were kept into liquid nitrogen before storing them in the -80 °C freezer. Vitellogenin determination was performed using a direct Enzyme-Linked Immunosorbent Assay (ELISA) adapted from Pateraki and Stratakis <sup>[14]</sup>. The 96-well microtiter plates were coated with the standard solutions, purified VTG (0, 22, 10, 20, 50, 75 and 100 ng 100 µL<sup>-1</sup>) and haemolymph samples from each crab (200 µL). A polyclonal antibody raised in rabbits against *C. maenas* VTG could identify vitellogenin concentrations. The plate was read at 405 nm and VTG standards were fit to a linear regression ( $R^2 = 0.96$ ; slope = 0.144). 28-day VTG results were normalized with the 0-day VTG concentrations ( $[VTG^*] = [28\text{-days VTG}] - [0\text{-days VTG}]$ ) providing the amount of proteins that fluctuates during 28 days of exposure.

## 2.4. Statistical analysis

Contamination and VTG\* data were linked by factor analysis, using principal components analysis (PCA) as the extraction procedure (STATISTICA®); this is a multivariate statistical technique for exploring variable distributions. The objective of PCA is to derive a reduced number of new variables as linear combinations of the original variables. This provides a description of the structure of the data with the minimum loss of information [9].

## 3. Results and discussion

### 3.1. Chemical analysis

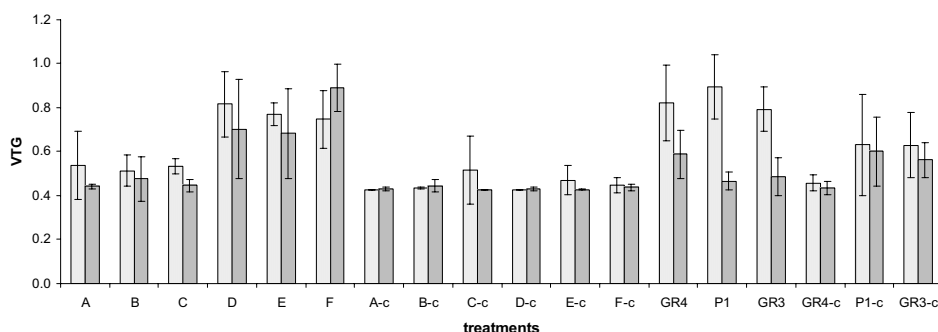
Sediments were mainly contaminated by PAHs, and the samples from the Bay of Algeciras, continuously affected by oil spills, turned out to be the most contaminated by these compounds (Table 1). On the other hand, results do not show a prevailing tendency in the concentration of metals among sediments from the different areas. High levels of Zn were detected in the stations A, C (Cies Island) and F (Corme-Laxe) from Galicia, whereas Cu levels were high P1 and Ni in GR3, both stations located in the Bay of Algeciras.

**Table 1.** Values of total organic carbon (%dry weight), fines (% of dry sediment < 63 mm) and the concentration of contaminants (metals (mg kg<sup>-1</sup> dry weight);PAHs and PCBs (mg kg<sup>-1</sup> dry weight)) in sediment samples from Galicia (Cíes Island: A, B, C; and Corme-Laxe: D, E, F) and Algeciras Bay (GR3, GR4 and P1). Not detected is expressed by n.d.

<b>Stations</b>	<b>O.C.</b>	<b>Fines</b>	<b>Zn</b>	<b>Pb</b>	<b>Cu</b>	<b>Ni</b>	<b>Co</b>	<b>V</b>	<b>PAH</b>
<b>A</b>	0.28	4.32	377	1.50	5.20	13.3	0.30	0.70	108
<b>B</b>	0.26	2.81	91.0	0.90	1.40	2.40	0.20	0.80	67.0
<b>C</b>	0.30	2.76	164	0.85	1.40	4.50	0.10	0.60	n.d.
<b>D</b>	0.31	3.79	25.0	3.70	0.70	1.70	0.34	2.00	38.0
<b>E</b>	0.37	5.50	19.9	7.30	0.43	1.50	0.35	2.10	52.0
<b>F</b>	0.65	5.95	271	5.90	4.20	5.70	0.36	3.40	323
<b>GR3</b>	2.15	69.4	138	21.6	5.01	74.7	12.8	26.1	3150
<b>GR4</b>	3.19	59.3	35.3	6.21	3.67	13.1	5.59	n.d.	802
<b>P1</b>	3.86	35.4	56.7	12.3	75.2	13.3	n.d.	6.84	641

### 3.2. Vitellogenin analysis

Results of vitellogenin concentration in haemolymph decreased in the majority of the treatments after 28 days of exposure (Fig. 2). The decline was detected mainly in the laboratory tests whereas caged crabs presented a lower variation in the vitellogenin levels after the exposure period. This could be due to the fact that under caged crabs in field were subjected to the environmental conditions such as changes in the variables and currents what may decrease the availability of the contaminants. Organisms exposed to sediments from the Bay of Algeciras (GR3, GR4 and P1) suffered the highest variations in vitellogenin levels.



**Figure 2.** Levels of vitellogenin (ng 100mL<sup>-1</sup>) in haemolymph of crabs exposed to sediments from Galicia (Cíes Island: A, B, C; and Corme-Laxe: D, E, F) and Algeciras Bay (GR3, GR4 and P1); samples from the caged organisms have the suffix “-c”. The results shown match with the day 0 (dotted bar) and day 28 (striped bar) of exposure.

### 3.3. Statistical analysis

The variables (O.C., fines, Zn, Pb, Cu, Ni, Co, V, PAHs, and VTG) were autoscaled (standardized) so as to be treated with equal importance [15]. The application of the PCA to the original 10 variables indicates that they can be grouped in two new factors which explain a 72% of the total variance in the original data set. A group of variables as those associated with a particular component where the loading was 0.30 or higher was interpreted (Table 2). The first principal factor, #1 is predominant (50%) and it groups the variation of vitellogenin concentration in the haemolymph of the crabs with the presence of PAHs and the metals Pb, Ni and V in the sediment and its association with the organic carbon and grain size. Factor #2 (22%), shows the relationship between

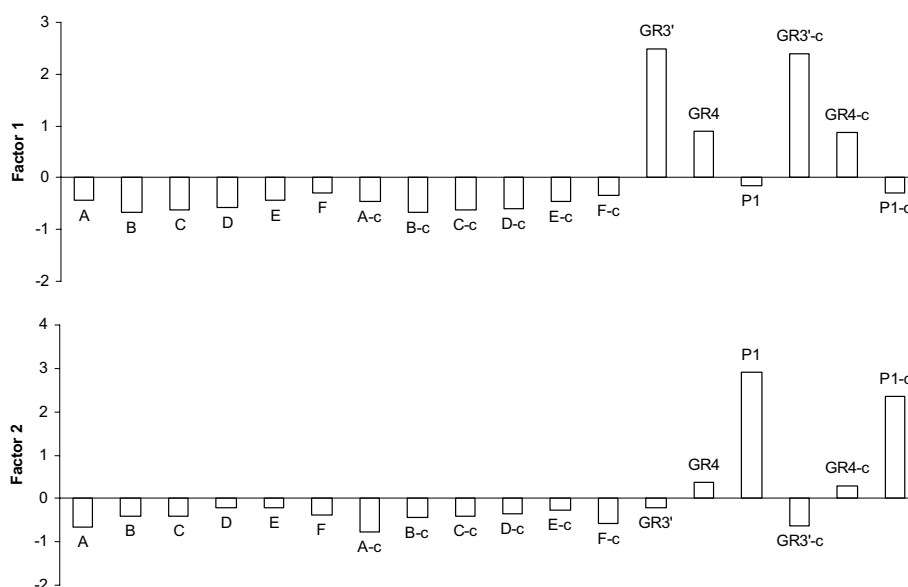
the grain size and the total organic carbon in the sediments with the presence of Pb, Cu and Co and the link with the variation in vitellogenin levels.

The influence of the two factors at the 18 treatments is reflected by the Factor score at these treatments and is shown in Figure 3. Factor 1 which shows the relationship of the vitellogenin variation and the contamination by PAHs, Pb, Ni and V has a main prevalence in the stations from the Bay of Algeciras GR3 and GR4 both in laboratory and field exposures. Factor #2, which links the variation of vitellogenin concentration with the metals Pb, Cu and Co, it is mainly prevalent in the stations GR4 and P1, laboratory and caged exposures, in the Bay of Algeciras. Treatments from the Galician Coast did not present positive loading in the factor scores.

**Table 2.** Sorted rotated factor loadings (pattern) of 10 variables for the two principal factors resulting from the multivariate analysis of results obtained from the chemical analysis and the vitellogenin determination.

	<b>FACTOR1</b>	<b>FACTOR2</b>
<b>Zn</b>	—	—
<b>Pb</b>	0.84	0.32
<b>Cu</b>	—	0.95
<b>Ni</b>	0.93	—
<b>Co</b>	—	0.95
<b>V</b>	0.54	—
<b>PAH</b>	0.97	—
<b>%C.O.</b>	0.53	0.80
<b>%fines</b>	0.91	0.33
<b>VTG</b>	0.37	0.56

Results obtained in the Statistical analysis have shown that the range of variation of the vitellogenin concentration in the haemolymph of the crabs was related to the PAHs and the metals Pb, Ni, Cu, Co and V. Although V and Co



**Figure 3.** Estimated factor scores for the two factors in each of the 18 cases. The factor scores quantify the prevalence of each factor for every station and is used to establish the definition of each factor. Samples from the caged organisms have the suffix “-c”.

are included in the definitions of factor #1 and 2 respectively, they appear in low concentrations in the sediments what means that probably the correlation is due to basal levels of these metals in the environment and do not suppose contamination. Previous studies showed a relationship between vitellogenin and metals <sup>[16]</sup>, although in that case there was a vitellogenin induction along the time of exposure, whereas in the present case of study vitellogenin decreased in the majority of the treatments. Other studies <sup>[17]</sup> suggested that metals may interfere with the ovarian cycle in *Carcinus maenas* and, therefore, with the reproduction of this species. Preceding investigations in fishes <sup>[18]</sup>, considered that low vitellogenin levels in females could be indicative of pollution induced dysfunction at the reproductive endocrine system level. Alterations in vitellogenin-like protein levels were observed in mussels exposed

to organic pollutants <sup>[19]</sup> whereas studies with female clams exposed to PAH contamination presented low levels of alkali-labile phosphate which positive correlates with vitellogenin <sup>[20]</sup>.

#### **4. Conclusions**

In the present study a decrease of vitellogenin concentration in haemolymph from the crab *Carcinus maenas* exposed to contaminated sediments was detected after 28 days of exposure. The variation of vitellogenin concentration was related to the presence of PAHs and the metals Pb, Ni and Cu in the sediment, which occurred mainly in the treatments from the Bay of Algeciras (chronically affected by oil spills) whereas the Galician Coast (acutely impacted by an oil spill) did not present this association. This points to a recovery of the area affected by the oil spill. Although both field and laboratory tests presented the same trends in vitellogenin variations, the response was lower under field conditions which means that laboratory tests resulted to be more sensitive than field studies in order to assess sediment toxicity.

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# **A multibiomarker approach using the polychaete *Arenicola marina* to assess oil contaminated sediments**

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## **Abstract**

Marine and coastal sediments can accumulate substantial concentrations of metals and hydrocarbons, yet the consequences of this contamination for exposed biota in situ can be difficult to establish. Here, we examine the hypothesis that exposure to contaminated sediments can lead to detrimental effects to sediment dwelling species. The common lugworm *Arenicola marina* was exposed in the laboratory for 14 days to marine sediments collected from sites of contamination in Spain and England. A suite of biomarkers of sublethal toxicity was combined with analytical chemistry to test for relationships between sediment contamination and effect. Moderate to strong correlations between organics, metals and biological responses were observed, with DNA

damage as measured using the Comet assay forming the largest contribution towards the observed differences. The response of worms from sites experiences different contamination loads were clearly distinguishable. The use of *A. marina* in this way provides a sensitive, holistic approach to sediment toxicity assessment, enabling comparisons between chronically, and acutely polluted sites to be quantified and recovery of these sites to be charted.

## **1. Introduction**

An integrated approach to marine pollution monitoring, that combines the traditional chemical analyses with laboratory and field based toxicity testing, is becoming increasingly important in gaining better assessments of the pollution process in the marine and coastal environment (Chapman., 2007). The water framework directive (WFD) requires member states to assess the ecological quality status (EQS) of water bodies and to achieve “good water status” for all European waters by 2015 (EEC, 2000). Interest is therefore focused on developing assessment tools to monitor littoral ecosystems following the WFD requirements. Biomarkers have been shown to be useful tools in characterizing the health status of animals from impacted areas, where complex mixtures of pollutants are usually present (Galloway et al., 2002; Galloway et al., 2004). The combination of chemicals and biomarkers as part of a weight of evidence (WOE) approach allows the identification of the impact of chemical contamination on different levels of biological function and could make a viable addition to routine management protocols for protecting the environment (Galloway et al., 2004) but has rarely been achieved for sediment dwelling species. Since many persistent organics and metals are retained within sediments, this represents a major knowledge gap in ecotoxicological monitoring programmes of the marine environment.

*Arenicola marina* is a common intertidal polychaete which is highly suitable for the biomonitoring of sediment-bound contaminants: it lives in U-shaped burrows within the sediment and ingests large volumes of sediment when feeding, therefore is continuously exposed to any contaminants present in the sediment. It is available all the year round, often in reasonably high densities, tolerates a wide range of particle sizes and salinities and has a broad geographic range (Bat et al., 1998). *Arenicola marina* is also an important link in coastal food chain playing an important role in sediment community organization (Bat et al., 1998). Polychaete worms are often the most abundant taxa in contaminated areas and their capacity to accumulate and metabolize PAHs may have important effects on the transport and fate of PAHs in the marine environment (Selck et al., 2003). The study of water-soluble metabolites in *A. marina* highlights the presence of a PAH metabolising system in the organism (Christensen et al., 2002). During the past few years the 10-day acute sediment assay using *A. marina* has been widely adopted for use in evaluating the quality of sediments (CEFAS, 1998; Thain and Bifield 2002). Although this bioassay supplies information about general health, it does not clarify how or why the organisms are affected. Biomarkers studies in *Arenicola marina* are few, (Hannam et al., 2007; Lewis et al (in prep)), however, some specific and non-specific biomarkers have been investigated in other polychaete species (for example; *Nereis diversicolor*, Durou et al., 2007); *Capitella capitata*, Bach et al., 2005); *Laeonoreis acuta*, (Montserrat et al., 2006); *Tubifex tubifex*, Mosleh et al., 2007); *Sipunculus nudus*, Matozzo et al., 2002). To date, these techniques have not previously been combined to give a multi-biomarker approach to sediment quality monitoring using a single species.

In the present study, we use a novel approach to sediment toxicity assessment, which combines a multi-biomarker approach using an *Arenicola* exposure model with analytical chemistry to address the hypothesis that

exposure to contaminated sediments can cause detrimental biological effects. Marine sediments were collected from sites around Europe exhibiting varying degrees of anthropogenic impact and included sites recovering from the acute impacts provoked by the tanker *Prestige* (2002) in the Galician Coast (NW Spain) (Morales-Caselles et al., accepted) and an area chronically affected by oil spillage. Specific questions included: (1) can a significant relationship between contamination and biological response be shown? (2) can we use this integrated approach to distinguish between acutely and chronically impacted sites? (3) Is this technique sensitive enough to chart recovery?

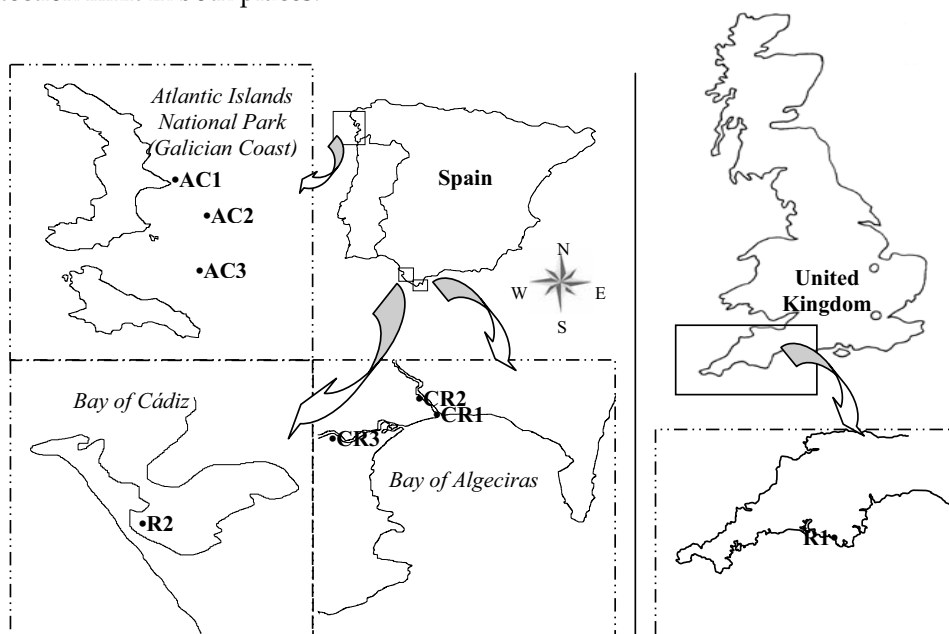
## **2. Materials and methods**

### **2.1. Study site**

Contaminated sediments from two areas of the Spanish coast were selected. The Atlantic Islands National Park is located in front of the mouth of the Rias Baixas in the Galician Coast (NW Spain) and it is considered a place with high ecological relevance. These islands played an important role in the *Prestige* oil spill (November, 2002) acting as a barrier that protected the coast from the entrance of spilled oil. Sediments from the Bay of Algeciras (S Spain) have suffered a chronic impact lasting several decades, caused by the input of oil and other contaminants from the various industries located in the area and from accidental spills and deliberate discharges from commercial shipping activities (Morales-Caselles, et al 2007).

To perform this study 3 stations were selected in the National Park and 3 stations in the area of the Bay of Algeciras (Figure 1). Two reference sites were selected to carry out this study: in the Estuary at Exmouth, South Devon, U.K, and in the Bay of Cádiz, Spain. The first was the site of collection of *Arenicola marina* specimens whereas the second was chosen because it has been widely characterized in previous ecotoxicological studies (DelValls et al., 1998,

Morales-Caselles et al., 2007); in addition, organic contamination was below the detection limit in both places.



**Figure 1.** Map of general areas sampled and locations of the sampling stations in Galicia (NW Spain) and the Bay of Algeciras (S Spain), both affected by oil spills, and the reference sites located in the Bay of Cádiz (S Spain) and Exmouth (S UK).

## 2.2. Sample collection and bioassay

Sediment samples were collected and transported to the laboratory and sub-sampled for physical characterization and chemical quantification. After that, sediment samples were maintained in the cooler at 4° C in the dark until they were used for sediment toxicity testing, but for no longer than 2 weeks. *Arenicola marina* specimens were obtained from a natural population from a 'clean' (<http://www.environment-agency.gov.uk/>) estuary at Exmouth, South Devon, U.K (50°36'57"N 3°26'40"W). Organisms were placed in 20 L capacity aquariums with clean, filtered (0.5µm) seawater (FSW) and sieved sediment

(collected in the same area as the organisms) and were maintained in laboratory under controlled conditions for acclimation (7 days) until the start of the test. Aeration was provided with a 12:12 light: dark photoperiod.

The toxicity test was conducted in replicate (5) by exposing individual *Arenicola marina* specimens to bulk sediment. Approximately, 250 g of sieved (1 mm) sediment was placed in 2 L beakers with 750 mL of well aerated FSW. Two *A. marina* were placed in each replicate container and maintained at 15 °C during the 14 day exposure period. The behaviour and casts assay was performed after the exposure period. after which coelomic fluid was collected for use in the cellular assays. Coelomic fluid was carefully withdrawn from the posterior part of each *A. marina* specimen using a 21G syringe, and stored in ice prior to use. The whole body was then frozen at -80 °C for subsequent biochemical biomarkers analysis. Worms were homogenized with PBS pH 7.5 and centrifuged for 30 minutes at 10,000 g at 4 °C; supernatant was employed for biochemical biomarkers and total proteins determination (Bradford et al., 1976).

*Chemical analysis.* The analyses of PAHs and PCBs bound to sediments were carried out according to USEPA SW-846 Method 827C78082 (1994). Briefly dried samples were Soxhlet extracted with n-hexane for 18 h, and the extracts were isolated by column chromatography on Florisile alumino-silica. PCBs and PAHs were eluted and their fractions were dried in a rotating evaporator and re-dissolved in isooctane. Aromatic fractions were analyzed on a HewlettePackard (HP) 5890 Series II gas chromatographer coupled with an HP 5970 mass spectrometer. PAHs were analyzed by GC-MS using selected ion monitoring (SIM). Analysis of PCBs as AROCLOR 1242 and AROCLOR 1260 was performed using the same instrument with an electron capture detector (GC/ECD). For both set of organic chemicals, PAHs and AROCLOR, the

analytical procedure showed agreement with the certified values of more than 90%.

Trace metal analysis were analyzed as described by Casado-Martínez et al. (2006); briefly, 2.5 g of sediments (<0.065 mm) were placed in Teflon containers and were digested in microwave (400W, 15 min, twice) with HNO<sub>3</sub> 2N. The extracts were purified by passing through a C-18 column and metals analyses were performed by anodic voltamperimetry (-Zn, Pb, Ni, Co and Cu- Metrohm Application Bulletin N° 147; - V- Metrohm Application Note N° V-81). For Hg the cold vapour technique was used and was quantified using atomic absorption spectrometry. The analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) and showed a recovery greater than 90% of the certified concentration.

*Behaviour and Feeding assay.* After 14 days of exposure the individual *A. marina* specimens were transferred to different beakers containing clean sediment (from the reference site at Exmouth) and the time taken for them to re-bury themselves completely was recorded. Worms were returned to the test sediment beakers and left for 24 hours; after which casts were carefully removed, dried and weighed.

*Comet assay.* The Comet assay was performed to detect single strand DNA breaks in individual cells according to the methods of Singh et al. (Singh, 1988) with modifications specific for *Arenicola marina* (Lewis et al., in prep), using alkaline conditions. One hundred cells per preparation were quantified using Kinetic COMET Software.

*Phagocytosis.* Phagocytosis activity was determined by measuring the uptake of fluorescent zymosan particles, using trypan blue as a quenching agent Anderson et al (1995). In brief, 50 µL of coelomic fluid was pipetted in triplicate wells of a microtitre plate; then 50 µL Fluorescein isothiocyanate (FITC) was added to the wells. Incubation was performed in dark for 40 min, at

21 °C. 50 µL of Fluorescence quenching solution (1.25 mL trypan blue in 1 mM citrate buffer pH 4.5) was then added to the wells, and fluorescence measured using a Hitachi F-4500 fluorescence spectrophotometer  $\lambda_{\text{ex/em}}$  485/535 . Results were compared to a standard curve and normalised to protein.

*Antioxidant status.* Antioxidant status was measured using the ferric reducing ability of plasma (FRAP) assay (Benzie and Strain, 1996) as adapted by Hagger et al., 2005. Coelomic fluid, 50 µL, in duplicate was incubated for 10 min at 25°C with 200 µL of FRAP reagent (300 mM acetate buffer pH 3.6, 2,4,6-tripyridyl-5-triazine (TBTZ), 20 mM iron chloride in the ratio 10:1:1 prepared immediately prior to analysis) in microtitreplates and the change in absorbance at 593nm noted. The FRAP value was calculated relative to a standard curve of Fe(II) in the range 100-500 µmol/l and expressed as change in absorbance per mg protein.

*Thiobarbituric acid reactive substances (TBARS) assay.* The measurement of TBARS was performed to evaluate the free radical-mediated oxidation (modified by Camejo et al., 1999). Malondialdehyde (MDA), a secondary product in lipid peroxidation binds to thiobarbituric acid (TBA) which can be measured spectrophotometrically. Briefly, 10 µL of free radical scavenger 1 mmol L<sup>-1</sup> butylated hydroxytoluene (2,6-Di-*tert*-butyl-4-methylphenol) dissolved in absolute ethanol was added to the microplate wells in order to prevent further oxidation of the samples; 40 µL of homogenate and 200 µL phosphate buffered saline pH 7.4 were added to the wells. 50 µL of 50 % (w/v) trichloroacetic acid and 75 µL of 1.3 % (w/v) thiobarbituric acid (TBA) (dissolved in 0.3% (w/v) NaOH) were included and after 60 min at 60 °C incubation the absorbance was read at 530 nm and then again at 630 nm. Results were compared to a standard curve prepared using 1,1,3,3-tetraethoxypropane (a stabilized form of MDA) and normalised to protein.

*Glutathione transferase (GST) assay.* The phase II metabolizing enzyme Glutathione-S-transferase (GST) was determined by monitoring the rate of conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (McFarland et al.,1999). Supernatants were diluted (20 µL in 1 mL homogenizing buffer) and placed in the 96 wells plate. 0.5 mL 42 mM GSH were added to a mixture containing 0.5 mL 42 mM CDNB (in ethanol) and CDNB assay buffer (200 mM sodium phosphate, pH 6.5); immediately 200 µL of the solution were placed into the wells and the plate was read at 340 nm every 30 seconds for 3 minutes.

*Glutathione Reductase (GR) assay.* The oxidation of 1 mM NADPH by GR in the presence of 10 mM oxidized glutathione was monitored at 340 nm (McFarland et al.,1999). In brief, 20 µL of supernatant was pipetted in triplicate wells of a microtitre plate. A solution containing 2.5 mL 10 mM oxidized glutathione, 2.5 mL 1mM NADPH and 20 mL GR buffer (200 mM sodium phosphate, pH 7.6) was prepared and 200 µL were added to the wells. Plate was read at 340 nm every 2 min for 10 min

*Glutathione Peroxidase (GPX) assay.* The antioxidant GPX activity was measured according to (McFarland et al.,1999). Supernatants were diluted (10 µL + 10 µL homogenizing buffer) and placed in the 96 wells plate. A solution containing in excess NADPH, reduced glutathione and glutathione reductase was prepared and 200 µL were added to the wells. After 2 min incubation 50 µL of 1.25 mM hydrogen peroxide was pipetted to the wells; NADPH oxidation was measured at 340 nm at 10 s intervals for 3 min.

*Statistical analysis.* Data for each biomarker were analyzed using ANOVA in order to determine significant differences ( $p < 0.05$ ) among the results obtained in each collection site and the reference site. Correlation between chemical concentrations in sediments and biomarker responses was carried out using a Spearman correlation analysis ( $p < 0.05$ ). Multivariate analyses were also

performed using the MDS, SIMPER, ANOSIM and BIOENV programs of the PRIMER software package (Plymouth Marine Laboratory, UK). A Bray-Curtis dissimilarity matrix was produced from fourth root transformed raw abundance data. Non-metric Multi-Dimensional Scaling (nMDS) was then performed to produce two-dimensional ordination plots. In ordination plots, points (sites) close to each other have similar biomarker responses, whilst those far apart are less similar. One-way ANOSIM tests were used to test for significant differences between biomarker responses in reference, chronically polluted and acutely polluted (i.e. Prestige) sites. Similarity percentages (SIMPER) were then used to identify the percentage contribution of each biomarker to the multivariate differences between the different sites (Clarke and Warwick, 1994) and the BEST (BIO-ENV) programme was used to determine which chemical parameters measured 'best' describe the pattern in biomarker responses observed in *Arenicola marina* specimens exposed to the different sediments.

### **3. Results**

#### **3.1. Chemical analysis of sediments**

The concentration of organic contaminants (PAHs) was much higher in sediments collected in the Bay of Algeciras (CR2 > CR1 > CR3) than in the area of Galicia (AC3 > AC2 > AC1), whereas the concentration of metals did not present a clear trend among sediments from the different areas (Table 1). The references (R1 and R2) presented the lowest levels of metals, and no organic contamination was detected. The predominant PAH in the sediments collected in the National Park was the naphthalene whereas sediments located in the Bay of Algeciras mainly presented phenanthrene, fluorene, pyrene and benzo[b]fluoranthene.

**Table 1.** Total PAHs, PCBs and metal concentration (Zn, Cd, Pb, Cu, Ni, Co, Hg and V) -mg Kg<sup>-1</sup> dry sediment-, percentage of fines (fines) and organic carbon (O.C.) measured in the sediments. n.d: not detected.

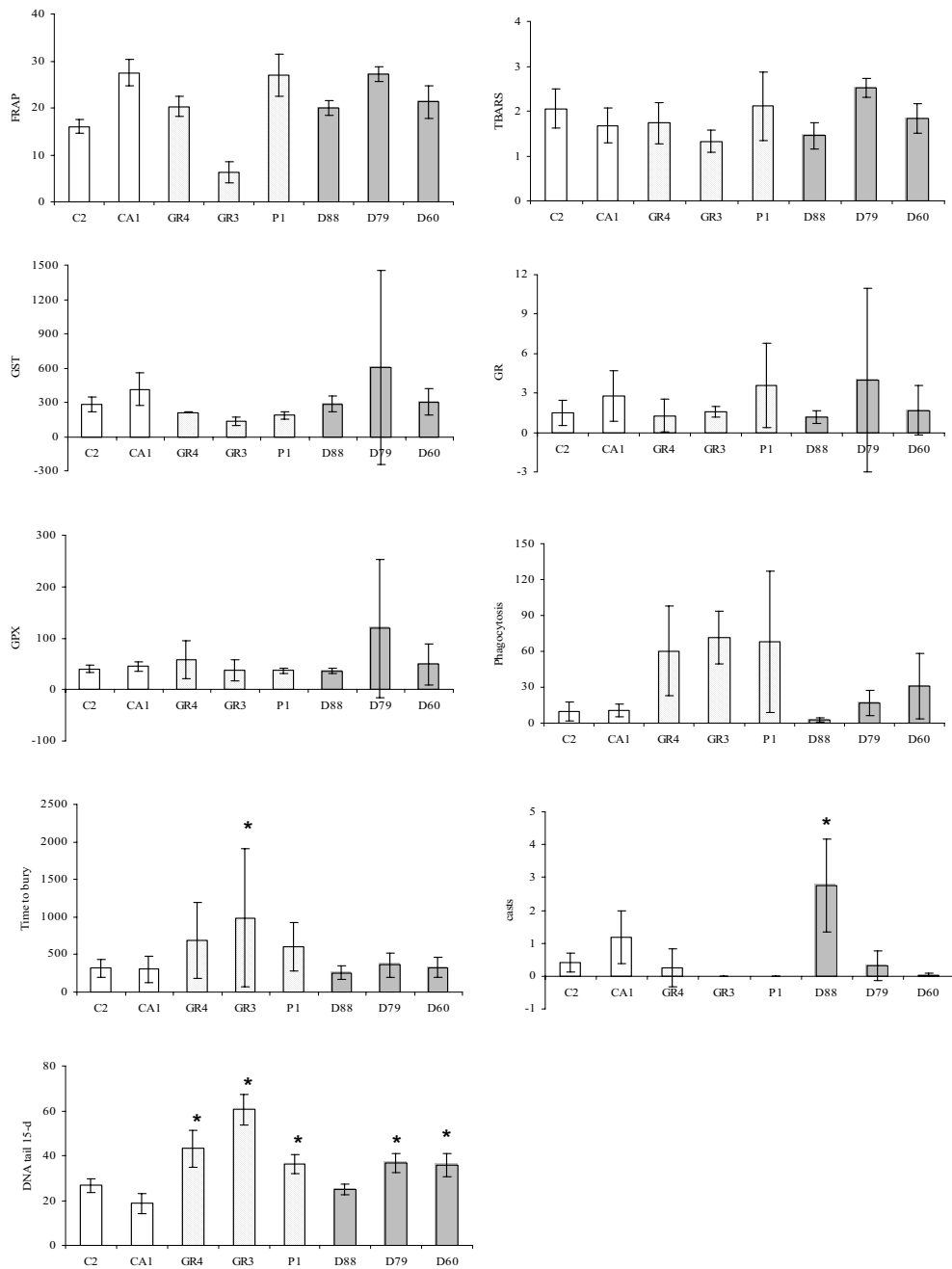
	Zn	Cd	Pb	Cu	Ni	Co	V	Hg	PAH	PCBs	O.C.	fines
<b>C2</b>	12.4	0.06	11.6	17.6	13.1	1.8	3.27	0.15	n.d.	n.d.	1.22	22.7
<b>Ca1</b>	21.3	0.92	2.28	6.98	0.06	3.40	80.0	n.d.	n.d.	n.d.	1.07	2.50
<b>GR4</b>	35.3	0.10	6.21	3.67	13.1	5.59	n.d.	0.25 <sup>a</sup>	802 <sup>b</sup>	1.75	3.19	59.3
<b>GR3'</b>	138	0.17	21.6	5.01	74.7 <sup>a</sup>	12.8	26.1	1.04 <sup>a</sup>	3151 <sup>b</sup>	22.0	2.15	69.4
<b>P1</b>	56.7	0.12	12.3	75.2 <sup>a</sup>	13.3	n.d.	6.84	0.65 <sup>a</sup>	641 <sup>b</sup>	0.84	3.86	35.4
<b>D88</b>	158 <sup>a</sup>	n.d.	17.3	20.1	12.4	n.d.	n.d.	0.28 <sup>a</sup>	13.0	n.d.	0.26	2.35
<b>D79</b>	107	n.d.	21.0	39.1 <sup>a</sup>	21.1 <sup>a</sup>	0.30	n.d.	0.09	80.0	n.d.	2.08	65.2
<b>D60</b>	161 <sup>a</sup>	n.d.	43.4	16.7	14.7	0.20	n.d.	0.12	260	n.d.	2.07	50.0
<b>SQVs</b>	150	1.2	46.7	34	20.9	-	-	0.15	624 <sup>*</sup>	22.7	-	-

Chemical data was compared to international sediment quality guidelines (SQGs) that specify the levels of chemical contaminants associated with biological effects and those exceeding recommended limits are highlighted in Table 1. Following the recommendations described by MacDonald et al. (1996), the sediments from the Bay of Algeciras would be considered as slightly polluted by PAHs and adverse effects might be predicted.. Site AC3 also present a naphthalene content higher than the ERL (160 µg kg<sup>-1</sup>) proposed by NOAA (1999) (ERL: values below which biological effects are rare). In the Bay of Algeciras, site CR2 surpassed the ERL defined for fluorene (19 µg kg<sup>-1</sup>) with high values of phenanthrene and fluoranthene. CR3 from the Bay of Algeciras exceeded the guideline for Cu, as did AC2 in Galicia. Zn ERL value is surpassed by the sediments AC1 and AC3; Ni sediment concentration goes above the ERL defined in CR2 and AC2 whereas Hg SQG is surpassed by sediments from the Bay of Algeciras and the treatment AC1 located in the

Galician Coast. PCBs were only detected in samples from the chronic sites although levels were below the SQGs.

### 3.2. Biomarker responses

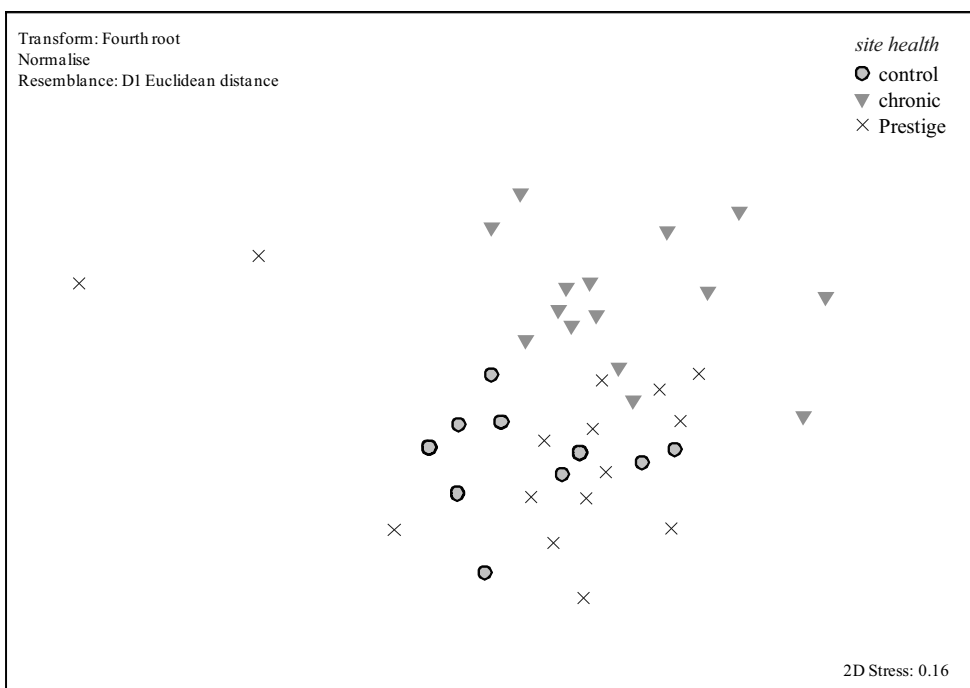
A summary of all the biomarkers results measured in *Arenicola marina* is shown in Figure 2. Comet results obtained after 15 days of exposure confirm significantly differences ( $p<0.01$ ) between the reference sites (R1 and R2) and the stations from the Bay of Algeciras CR1, CR2, CR3 and the sites AC2 and AC3 located in the AINP. In the Behaviour assay CR2 was significantly different ( $p<0.05$ ) to the R1, R2 and AC1 treatments whereas the weight of casts showed that AC1 was significantly different ( $p<0.01$ ) to all the stations, including the reference treatments. Differences were statistically significant for the phagocytosis assay although responses presented a high increase in variation. No significant differences were detected in FRAP, TBARS, GPX, GST and GR by using the ANOVA. In general, treatment AC1 from the Galician Coast and both reference sites present analogous trends. In contrast, sites from the Bay of Algeciras generally present more marked effects in the inhibition or increasing of the analyzed biomarkers, especially site CR2.



**Figure 2.** General health biomarkers: glutathione peroxidase GPX (nmol/min/mg prot), glutathione transferase GST (nmol/min/mg prot), glutathione reductase GR (nmol/min/mg prot), thiobarbituric acid reactive substances TBARS (nmol/mg prot), ferric reducing ability of plasma FRAP

( $\mu\text{M}/\text{mg}$ ), phagocytosis (zymosan per mg protein  $\cdot 10^6$ ), behaviour assay (s), casts assay (g) and Comet assay (% DNA in tail) after 7 and 15 days of exposure.

Comparing the biomarker responses between the different sites using the multivariate ANOSIM (Primer 6 software) demonstrated a significant effect of chronic pollution on the biomarker responses of *Arenicola marina* (Figure 3). An *a priori* one-way ANOSIM comparing the biomarker responses of *Arenicola marina* exposed to 'clean' reference sediments with those exposed to sediments from chronically polluted and acutely polluted (*Prestige*) sites, reveals a significant difference in biomarker response between the chronically polluted sites and the other sites ( $R = 0.281$ ,  $P = 0.001$ ). No significant difference was observed between the *Prestige* affected sites and the two reference sites (Figure 3). Similarity percentages (SIMPER) were used to identify the percentage contribution of each biomarker to the multivariate differences between all sites (Clarke and Warwick, 1994). The comet assay was found to make the largest contribution to the observed differences between the reference sites and the chronically polluted sites, representing 21.62% of the dissimilarity between sites, whilst the burrowing assay made up 14.10% of the dissimilarity between the *Prestige* sites and the chronically polluted sites.



**Figure 3.** Two dimensional non-metric multidimensional scaling plot of the biomarker responses for each *Arenicola marina* specimen exposed to sediments from the different experimental sites: representing 'clean' control sites; sites affected by chronic pollution and sites affected by the Prestige oil spill (i.e. an acute pollution incident).

### 3.3. Linking chemicals and biomarkers

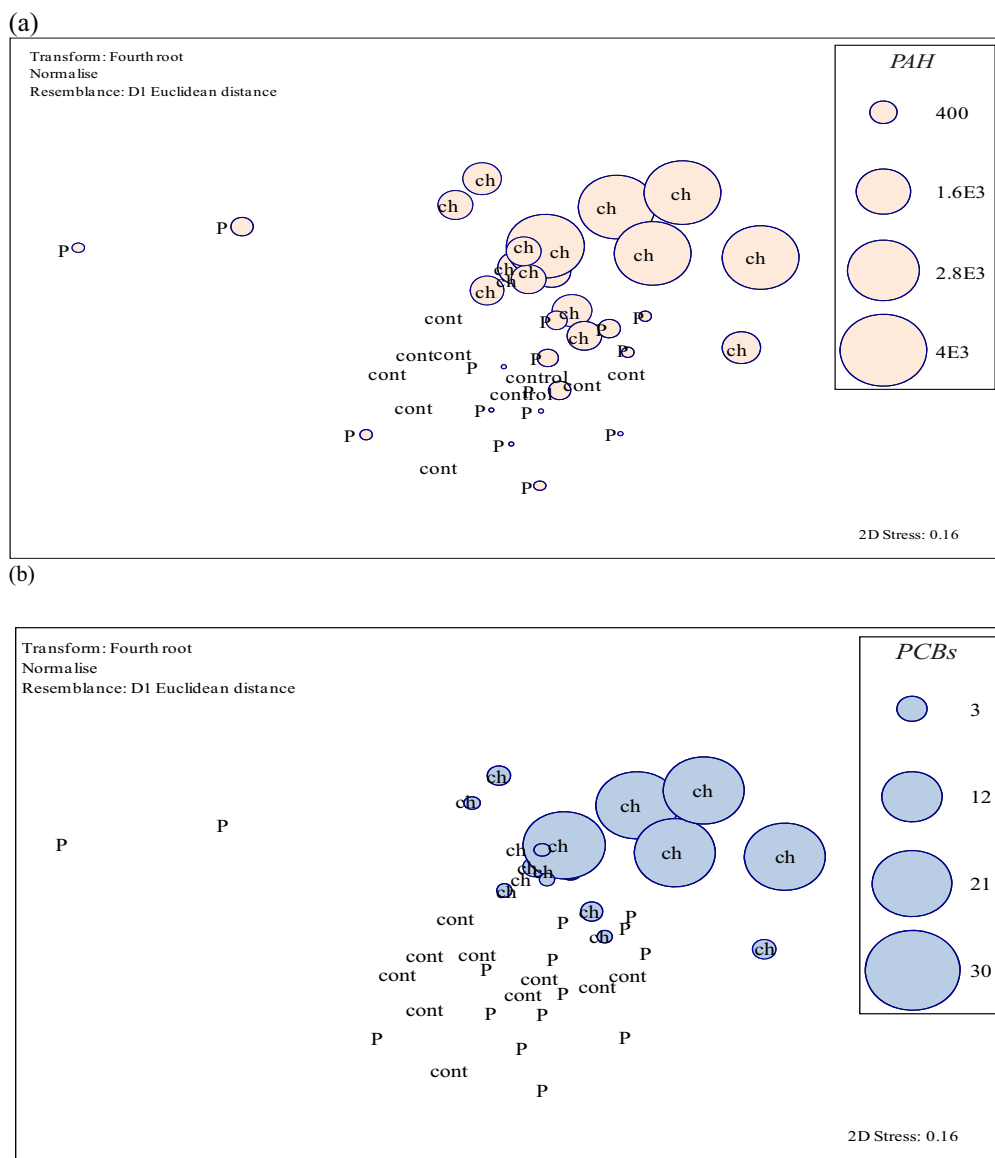
The chemical parameters measured that best account for the pattern observed in the biomarker responses of *Arenicola marina* are the sum of PAHs and the PCBs (Figure 4, BEST analysis, Spearman's Rank correlation coefficient 0.361). A strong positive relationship was observed between organic contaminants (PAHs and PCBs) with the DNA damage (0.89, 0.79, respectively), the phagocytic response (0.90, 0.87, respectively) and the burial behaviour (0.90, 0.85, respectively). A negative correspondence was detected with the weight of

casts. On the other hand, the phase II detoxification enzyme GST presented a negative relationship with the organic contaminants, whereas no correlation was found with the antioxidants enzymes.

#### **4. Discussion**

The results demonstrate how a combination of multi-biomarkers with analytical chemistry can be used to investigate the toxicity of marine sediments, enabling the differentiation of sites showing different types of contamination. There are clear relationships in sublethal assays that can be related to the putative mode of toxicity of the contaminants.

Phenanthrene, fluorene, pyrene and benzo[b]fluoranthene are the predominant PAHs in sediments from the Bay of Algeciras which presented the highest impact in the organisms exposed. These compounds are considered priority pollutants by the USEPA on the basis of their toxicity, frequency of occurrence and potential for human exposure (Ming-Ho Yu, 2005). It is known that PAHs affect organisms through toxic action by the interference with cellular membrane function and induction of enzyme systems associated with the membrane (Albers, 2003). Although unmetabolized PAHs can have toxic effects, a major concern in animals is the ability of the reactive metabolites, such as epoxides and dihydrodiols, of some PAHs to bind to cellular proteins and DNA (Albers, 2003). This can explain the elevation in DNA damage detected in those organisms exposed to sediments from the Bay of Algeciras. The DNA damage observed in the lugworms links to the results obtained in the behaviour and casts assays confirming the effects of the toxicants on the metabolism and conduct. These results are in accord with previous studies which used other marine species and demonstrated that comet assay is a sensitive tool for monitoring the genotoxic effects of PAHs (Pérez-Cadahía et al., 2004).



**Figure 4.** Two dimensional non-metric multidimensional scaling plots for biomarker responses in *Arenicola marina* for the control sites (cont); chronically polluted sites (ch) and sites affected by the Prestige oil spill (P), overlaid with circles proportional in diameter to the concentration at each site of (a) PAH's and (b) PCB's.

Exposure to PAHs can lead to the formation of reactive oxygen species (ROS) which can also affect immune function through lipid peroxidation and membrane destabilisation of haemocytes (Di Giulio et al., 1989, Galloway and Goven, 2007). This concurs with observations of phagocytic activity from previous work with invertebrate species (Komiyama et al., 2003). The correlation between organic contaminants with the time of burrowing in the sediment suggests a chemosensory response, whereas the weight of casts decrease seems to be related to the feeding inhibition by the polychaete. PCBs have been linked with PAHs and similar relationship with biomarkers have been established. This points to a mixture of organic pollutants which are affecting the quality of sediments and suppose an environmental risk in the area of the Bay of Algeciras.

The metal content also showed correlations with the analyzed biomarkers. Ni shows a strong relationship with DNA damage (0.79), the phagocytic response, the burial behaviour and casts. Copper showed a relationship with the antioxidant activity analyzed in the TBARS assay, which measures one of the terminal products in the peroxidative breakdown of lipids. The induction of lipid peroxidation by copper is well-known in other invertebrates (Viarengo, 1989) and the TBARS activity has been previously associated with Cu (Quiniou et al., 2007). Weaker correlations were also detected among antioxidant biomarkers: FRAP associates with GST and GR whereas TBARS links with GR activity; however no relationships were observed for the GPX antioxidant enzyme with other biomarkers or pollutants. This suggests combined regulation of these responses.

After the Prestige oil spill investigations have addressed in determining the biological effects and environmental status after the accident by following

single lines of evidence, such as chemical analyses [(CSIC, 2003; Franco et al., 2006; González et al., 2006) and toxicity including biomarkers (Mariño-Balsa et al., 2003; Martínez-Gómez et al., 2006; Marigómez et al., 2006) however little has been done with the combination of both fields (Morales-Caselles 2006; Morales-Caselles, accepted2). In addition most of the biological assessments carried out towards the Prestige have been conducted in situ, under field; under controlled conditions in the laboratory, it is relatively straightforward to standardise biomarker assays and to regulate the chemical exposures that organisms receive, so that cause-effect and indeed, exposure-relationships, can be established (Astley et al., 1999).

Less attention than the played in the Prestige accident has been focused towards the chronic pollution of the Bay of Algeciras in recent years. The methodology employed in this study has shown how this area is much more polluted and biological effects on key invertebrates exposed to their sediments have been demonstrated. Other studies based on the Exxon Valdez oil spill suggested a recovery years after the episode and the prevalence of the chronic pollution due to the human and industrial activities (Boehm and Page, 2007).

Research methods based in biomarkers have not yet been validated for application as a monitoring method for oil spills in a systematic fashion (Boehm and Page, 2007). The selected biomarkers analyzed shows important relationships with pollutants and the proposed methodology which integrates different variables as part of a weight of evidence approach has demonstrated to be a suitable tool in oil spill assessments coming from different sources.

The bioassay performed with an invertebrate especie that lives in the sediment, the polychaete *Arenicola marina*, results to be relatively simple, rapid, economic and appropriate to test the environmental status of an oil-

contaminated sediment. This organism has been often used on monitoring programs; however, the application of suites of assays and chemistry are illustrated here for the first time to this polychaete. These have the potential to chart recovery after oil spills and have allowed the differentiation of sites with different types of contamination: the acutely (Prestige) and chronically (Bay of Algeciras) affected areas and the reference sites. The Comet assay has demonstrated to be the most sensitive of the studied endpoints, showing important correlations with the main contaminants. Authors consider the suitability of the using these tools in assessing environmental quality assessment and to chart recovery in areas affected by oil spills.

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## Capítulo 4.

### Evaluación de efectos subletales *in situ*

La calidad de los ecosistemas costeros se ha evaluado tradicionalmente siguiendo una metodología clásica que incluye el muestreo de especies autóctonas, el estudio de las comunidades bentónicas o la determinación de la toxicidad bajo condiciones de laboratorio (Burton Jr et al., 2005). Esta serie de estudios resultan muy útiles y en ocasiones esenciales, aunque presentan ciertas limitaciones (Tabla 1) (ej. Chapman et al., 1992; Burton et al., 1996; Grothe et al., 1996). El estudio de la toxicidad *in situ* mediante organismos en jaulas proporciona la información que falta en los estudios tradicionales (Burton Jr et al., 2005).

La ventaja principal de la exposición de los organismos a los sedimentos de estudio mediante el uso de jaulas radica en la obtención de una información sobre la toxicidad de los sedimentos evaluada bajo condiciones no controladas, que no sólo permiten estudiar la toxicidad producida por los contaminantes presentes en el sedimento, sino que permite evaluar el efecto producido por las variaciones fisicoquímicas a las que se ve sometido el medio y que pueden afectar a la disponibilidad de los contaminantes (Martín-Díaz, 2004). A pesar de las dificultades de la realización de aproximaciones *in situ* estas permiten llevar a cabo una evaluación más realista de los efectos biológicos producidos por contaminantes del medio. Además este tipo de estudios son capaces de

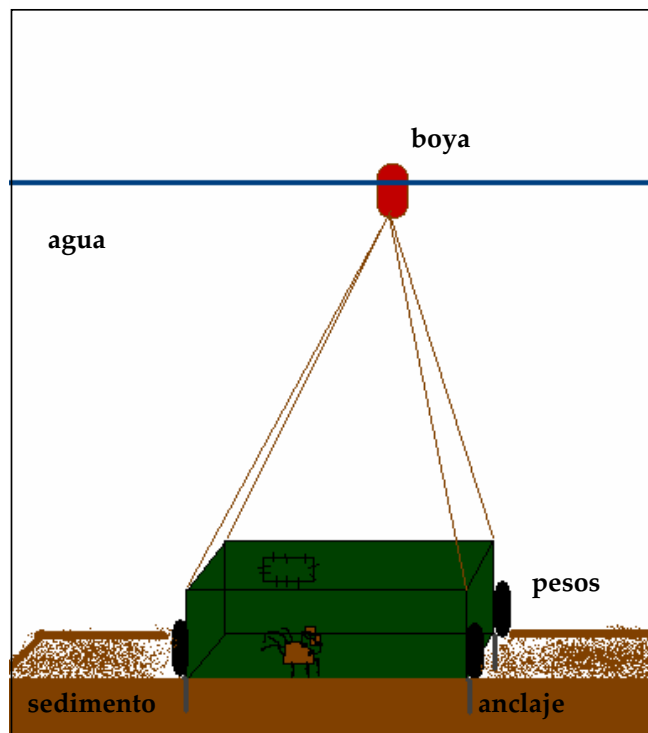
identificar fuentes de polución ajenas al sedimento pero que pueden ser la causa de alteraciones bentónicas en el medio, y no se pueden detectar mediante la consecución de experimentos de laboratorio aislados.

**Tabla 4.1.** Ventajas y limitaciones de los métodos tradicionales de evaluación de la calidad de ecosistemas costeros (según Burton Jr et al., 2005)

Metodología	Ventajas	Limitaciones principales
Guías Químicas	Fáciles y estandarizadas.	Extrapolaciones a campo y otras especies. Específicas de la zona. Basadas en estudios de laboratorio.
Biota autóctona	Ampliamente utilizada. Refleja los efectos. Interés público.	Variabilidad. Efectos indirectos. Causas naturales de estrés que dificultan interpretación.
Ensayos de toxicidad en laboratorio	Ampliamente utilizados. Integra efectos de contaminantes a corto plazo. Estandarizados.	Las condiciones no son iguales a las naturales en campo.
Bioacumulación	Exposiciones realistas. Útiles para elaborar modelos de redes tróficas. Medidas a largo plazo. Utilizadas tradicionalmente.	Metabolismo y excreción de algunos químicos. La aclimatación, adaptación y los metales esenciales pueden confundir la interpretación de los efectos observados.

En el capítulo 4 se presentan cuatro artículos, de los cuales el trabajo X, XI y XII muestran los resultados de medidas de biomarcadores tras exposiciones en campo de dos especies de invertebrados. En primer lugar, en el trabajo X se recogen los resultados de la instalación de jaulas en puntos de estudio del Golfo de Cádiz y la Costa de Galicia. En este estudio se seleccionaron dos especies de invertebrados marinos con hábitos distintos de alimentación, el cangrejo *Carcinus maenas* y la almeja *Ruditapes Philippinarum*. Se realizó una exposición de 28 días tras los cuales se llevaron a cabo medidas de biomarcadores de exposición (actividad EROD, GPX, GST y GR) y un biomarcador de efecto (histopatología). Este experimento se reprodujo bajo

condiciones de laboratorio como se explica en el Capítulo 3. Al comparar los resultados obtenidos en este experimento *in situ* con el descrito en el capítulo anterior desarrollado bajo condiciones de laboratorio, comprobamos como la inducción de los biomarcadores de exposición se daba mayormente en los organismos expuestos en jaulas ancladas en la Bahía de Corme-Laxe, en lugar de darse en los individuos localizados en la zona de Algeciras, mientras que los daños histopatológicos “se suavizaban” en las exposiciones en campo. Este hecho puede significar varias cosas: a) que, en general, los efectos biológicos bajo condiciones de campo son menores que en laboratorio, debido a la renovación continua de agua que disminuye la biodisponibilidad de los contaminantes; b) que las desembocaduras de los ríos Guadarranque y Palmones en Algeciras, sujetos a un importante régimen mareal, supongan una renovación mayor de agua; c) que dada la mezcla compleja de contaminantes no medidos en este estudio presentes en los sedimentos y posiblemente también en las aguas de la Bahía de Algeciras se den fenómenos de solapamiento entre la inducción/inhibición de los biomarcadores; d) que los factores abióticos afecten significativamente a la inducción de biomarcadores, principalmente en la desembocadura de los ríos en Algeciras; e) que la alta presencia de bateas en la Bahía de Corme-Laxe suponga un estrés a la biota debido a posibles sustancias contaminantes en piensos o a la alta carga orgánica del agua, y que expliquen por tanto, la notable inducción de los biomarcadores de exposición observados bajo condiciones de campo y que no fueron vistos tras los experimentos de laboratorio. Para completar este estudio se realizó una evaluación de la cinética de varios biomarcadores en la almeja *Ruditapes Philippinarum*, tal y como se muestra en el trabajo XI, aclarando de manera más efectiva las posibles fuentes de estrés; asimismo, se realizó un estudio cinético de las enzimas implicadas en la detoxificación de PAH en el cangrejo *Carcinus maenas* (trabajo XII).



**Figura 4.1.** Esquema de anclaje y utilización de jaulas bentónicas utilizadas en los bioensayos en campo.

El último trabajo de este capítulo, XIII, incluye una línea de estudio ajena a los bioensayos pero de gran importancia. En este trabajo se evalúa la alteración de la fauna bentónica de las áreas de estudio con el fin de relacionar los efectos de la biota autóctona con los contaminantes presentes en los sedimentos. De esta manera se cubre una de las líneas clásicas dentro de los estudios de calidad ambiental de los sedimentos. En este trabajo se observa como inicialmente la macrofauna bentónica de las costas gallegas se vio afectada por el vertido del petrolero *Prestige*, aunque se describe una recuperación importante que ha sido finalmente comparada con la situación de la biota de la zonas de estudio localizadas en la Bahía de Algeciras, donde el

impacto del conjunto de fuentes contaminantes supone un impacto ambiental mucho mayor.

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# ***Sublethal responses in caged organisms exposed to sediments affected by oil spills***

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## **Abstract**

The current study was performed to determine sublethal responses in two invertebrate species by using field deployments in areas affected by oil spills, acute in the Galician Coast (NNW, Spain) and chronic in the Bay of Algeciras (SSW, Spain). The organisms employed were the crab *Carcinus maenas* and the clam *Ruditapes philippinarum*., and during 28 days the animals were exposed in cages under field conditions to contaminated sediments. Different biomarkers of exposure were determined after 28-day exposure: ethoxyresorufin O-deethylase (EROD), phase I detoxification enzyme, glutathione-S-transferase (GST) phase II detoxification enzyme but also implicated in oxidative stress events, glutathione peroxidase (GPX) and glutathione reductase (GR), both antioxidant enzymes. In addition histopathological effects in target tissues of the deployed organisms were evaluated. Biomarkers measurements were linked with the concentration of chemicals in the sediments in order to elucidate the type, source and bioavailability of contaminants producing adverse effects in the bioindicator species. Results obtained in the present study have shown how the application of the selected battery of biomarkers under field bioassays allows identifying alternative sources of stress that are not possible to observe in laboratory experiments.

*Keywords: biomarker, histopathology, invertebrate, toxicity, contaminants*

## 1. Introduction

Measurements of an organisms' response to a pollutant at the biochemical or physiological level can detect more quickly and specifically the presence of toxic compounds, allowing earlier identification of change, before deleterious effects reach higher organization levels (Montserrat et al., 2003). Over the past decade, biomarkers have been used increasingly as diagnostic tools to investigate sublethal effects of toxic exposure and to elucidate the various modes of action of xenobiotics (De Coen et al., 2000). The application of biomarkers under field conditions has been proposed by many authors in order to assess chronic responses in aquatic populations exposed under environmental realistic conditions (Suter, 1993; Depledge and Fossi, 1994; De Coen et al., 2006; Martín-Díaz et al., in press). Field studies pose far greater difficulties due to the complex and fluctuating nature of the environment, and interactions among organisms within ecological communities. They address the integrated impact of anthropogenic and environmental stressors. Data collected in field studies may be much harder to interpret than data from controlled laboratory experiments (Astley et al 1999). It is also highlighted the potential use of *in situ* assays to determine the toxicity of sediments using different approaches including caging animals (Martín-Díaz, 2004). Sediment toxicity bioassays carried out in the laboratory are performed under strictly controlled parameters and thus do not reflect the variability in exposure that may occur in natural systems. This gives rise to uncertainty in the extrapolation of laboratory-based test results to natural environments in sediment risk assessment (Sibley *et al*, 1999).

In order to evaluate the exposure of contaminants related to oil spills in the organisms using *in situ* deployments and a biomarker approach, the objectives were as follows: (1) to test the feasibility of a suite of biomarkers to assess the oil-contaminated sediment quality (2) to identify the contaminants

bound to sediments which produce the sublethal effects in the organisms exposed (3) to determine the differences between the biological responses associated with a deployment to acutely and chronically oil contaminated sediments.

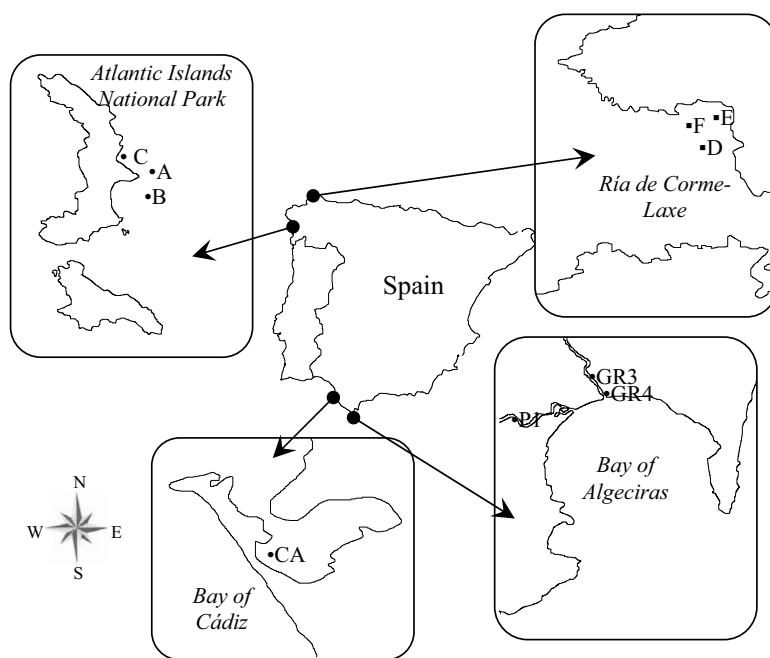
A battery of biomarkers of exposure of early biological effects was used in order to assess sediment toxicity of two coastal areas affected by oil spills, the Galician Coast, acutely impacted by the sinking of the tanker *Prestige* (2002) and the Bay of Algeciras chronically affected by several spills. Two invertebrate species with different feeding habits were selected to carry out the assessment, the crab *Carcinus maenas* and the clam *Ruditapes philippinarum*. The suite of biomarkers employed was: Ethoxyresorufin O-deethylase (EROD), phase I detoxification enzyme implicated in monooxygenation reactions of dioxins and PAHs; glutathione-S-transferase (GST) phase II detoxification enzyme but also implicated in oxidative stress events; glutathione peroxidase (GPX) and glutathione reductase (GR), antioxidant enzymes (Martín-Díaz et al., in press). Histopathological alterations in target tissues were also evaluated because it has shown to be responsive and sensitive to a wide range of contaminants and have been developed and recommended as biomarkers for monitoring the effects of pollution (Au, 2004).

## **2. Materials and methods**

### **2.1. Sites description**

The study was performed in two areas of the Spanish Coast: the Galician Coast (NW Spain) was chosen as it was affected by the *Prestige* oil spill in 2002, what supposed one of the major ecological catastrophes of the Iberian peninsula affecting more than 1000 km of coast; in this sense, the selected sites were located in the Cies Island in the Atlantic Island National Park and in the Bay of Corme-Laxe; the second area of study was the mouth of the River Palmones

and Guadarranque in the Bay of Algeciras (S Spain); this place was selected because is highly industrialized and there are a large number of petrochemical activities which comprise several accidental oil spills. A reference site was selected in a clean area in the Bay of Cádiz (S Spain) (Riba et al., 2004). The 10 selected study sites are shown in Figure 1: A, B, C located in Cíes, D, E, F in Corme-Laxe, GR3, GR4 and P1 in the Bay of Algeciras, and the reference site CA in the Bay of Cádiz widely characterized by different ecotoxicological studies (DeIvalls et al., 1998, Riba et al., 2004, Martín-Díaz et al., 2005)



**Figure 1.** Map of the coastal area of Galicia showing the locations of the sampling stations. A, B and C refers to the stations located in the Cíes Island in the Atlantic Island National Park and D, E and F to those in the Bay of Corme-Laxe. The stations located in the Bay of Algeciras are GR3, GR4 and P1. The station CA located in the Bay of Cadiz corresponds to the sediment used as reference.

## 2.2. Sampling and deployment

The clam *Ruditapes philippinarum* was obtained from an aquaculture farm whereas the crab *Carcinus maenas* was caught in a clean site located in the Bay of Cádiz (SW, Spain) (Riba et al., 2003). The organisms were transferred to the laboratory and kept in tanks with continuous water replacement under controlled conditions until the beginning of the experiment. The test animals were carefully transported to the study sites and placed in cages made with plastic mesh (50cm x 25cm x 15cm) divided in two different compartments, one for crabs (n=20) and one for clams (n=40). The cages were positioned with low tide and were wedged into the sediment. The exposure lasted 28 days during which crabs were fed once per week with mixed diet of mussels or fish. Sediment samples from the study sites were collected and transported to the laboratory where they were kept in dark at 4°C prior to chemical analysis.

## 2.3. Biochemical analysis

Deployed crabs and clams were collected and dissected after 28 days of exposure; hepatopancreas (in crabs) and digestive gland (in clams) were extracted and kept at -80°C prior homogenization. The samples were homogenized with Tris-acetate buffer following the procedure developed by Lafontaine et al. (2000). Samples were centrifuged at 10,000g for 30 min, and the supernatant was used for the biomarkers determination and the total protein content described by Bradford (1976). The phase II metabolizing Glutathione-S-transferase (GST) activity was determined by monitoring the rate of conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm, methodology adapted from McFarland et al. (1999). The oxidation of 1 mM NADPH by Glutathione Reductase (GR) in the presence of 10 mM oxidized glutathione was monitored at 340 nm, and the method was similarly adapted from McFarland et al. (1999). Mixed function oxygenase activity, which is the

first mode of detoxification of many organic pollutants, was measured using the EROD assay (Gagnè and Blaise 1993). The antioxidant enzyme Glutathione Peroxidase (GPX) was measured according to McFarland et al. (1999). Biomarkers results were normalized with the protein content.

#### 2.4. Biomarker of effect: Histopathology

Gills and digestive gland tissues of the organisms were fixed in phosphate buffered 10% formaldehyde (pH 7.2) for histopathology determination. After dehydration in graded concentrations of ethanol, the samples were embedded in paraffin wax. Histological sections of 6 to 8  $\mu\text{m}$  thickness were stained with Haematoxylin–Eosin and Haematoxylin–VOF [15]. Sections were reviewed by light microscopy Leitz Laborlux S and photographed (Sony DKC-CM30).

#### 2.5. Chemical analysis

The analyses of PAHs and PCBs bound to sediments were carried out according to USEPA SW-846 Method 827C78082 (USEPA, 1994). Briefly dried samples were Soxhlet extracted with n-hexane for 18 h, and the extracts were isolated by column chromatography on Florisile alumino-silica. PCBs and PAHs were eluted and their fractions were dried in a rotating evaporator and re-dissolved in isooctane. Aromatic fractions were analyzed on a HewlettePackard (HP) 5890 Series II gas chromatographer coupled with an HP 5970 mass spectrometer. PAHs were analyzed by GC-MS using selected ion monitoring (SIM). Analysis of PCBs as AROCLOR 1242 and AROCLOR 1260 was performed using the same instrument with an electron capture detector (GC/ECD). For both set of organic chemicals, PAHs and AROCLOR, the analytical procedure showed agreement with the certified values of more than 90%.

Trace metal analysis were analyzed as described by Casado-Martínez et al. (2006c); briefly, 2.5 g of sediments (<0.065 mm) were placed in Teflon containers and were digested in microwave (400W, 15 min, twice) with HNO<sub>3</sub> 2N. The extracts were purified by passing through a C-18 column and metals analyses were performed by anodic voltamperimetry (-Zn, Cd, Pb, Ni, Co and Cu- Metrohm Application Bulletin N° 147; - V- Metrohm Application Note N° V-81). For Hg the cold vapour technique was used and was quantified using atomic absorption spectrometry. The analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) and showed a recovery greater than 90% of the certified concentration.

## 2.6. Statistical analysis

The induction of biomarkers of response was analyzed with the ANOVA and Tukey test with the aim of determining significant differences ( $p < 0.05$ ;  $p < 0.01$ ) among the results obtained for the reference (CA) site and the other sampling sites, using the statistical package SPSS 11.5. Multivariate analysis was carried out with in an attempt to link contamination with adverse biological measurements; the principal component analysis (PCA) was used as the extraction procedure to derive a reduced number of new variables (factors) as linear combinations of the original variables (STATISTICA 6.0).

# 3. Results and discussion

## 3.1. Concentration of chemicals in the sediments

Results of the concentration of chemicals in the studied sediments are shown in table 1. The highest concentration of PAHs was found in the sediments from GR3 (2961 mg Kg<sup>-1</sup> dry sediment) located in the Bay of Algeciras, followed by sediments from the station F (820 mg Kg<sup>-1</sup> dry sediment) located in Corme-Laxe and GR4 (802 mg Kg<sup>-1</sup> dry sediment) and P1 (641 mg Kg<sup>-1</sup>

**Table 1.** Total PAHs, PCBs and metal concentration (Zn, Cd, Pb, Ni, Co and V) - mg Kg<sup>-1</sup> dry sediment- measured in the sediments from Galicia: Atlantic Islands National Park (A, B, C), Corme-Laxe (D, E, F); the Bay of Algeciras (GR3, GR4 and P1) and the Bay of Cadiz (CA) used as the reference station. n.d: not detected.

	PAHs	PCBs	Zn	Cd	Pb	Cu	Ni	Co	V
<b>CA</b>	n.d.	n.d.	21.3	0.92	2.28	6.98	0.06	3.40	80.0
<b>A</b>	257	n.d.	76.2	n.d.	26.6	18.9 <sup>d</sup>	12.0	0.52	n.d.
<b>B</b>	370	6.52	43.4	n.d.	9.13	n.d.	6.88	n.d.	n.d.
<b>C</b>	239	4.76	37.5	n.d.	6.54	31.6 <sup>d</sup>	5.02	0.87	n.d.
<b>D</b>	537	2.60	65.7	n.d.	44 <sup>d</sup>	22.1 <sup>d</sup>	9.39	1.21	13.4
<b>E</b>	558	4.29	31.8	n.d.	4.25	n.d.	5.61	0.37	2.34
<b>F</b>	820 <sup>d</sup>	2.28	243 <sup>a,b,d</sup>	n.d.	14.3	19.1 <sup>d</sup>	7.03	0.67	5.94
<b>GR3</b>	2961 <sup>d,e</sup>	22.0	138 <sup>d</sup>	0.17	21.6	5.01	74.7 <sup>a,d,e</sup>	12.8	26.1
<b>GR4</b>	802 <sup>d</sup>	1.75	35.3	0.10	6.21	3.67	13.1	5.59	n.d.
<b>P1</b>	641 <sup>d</sup>	0.84	56.7	0.12	12.3	75.2 <sup>a,c,d,e</sup>	13.3	n.d.	6.84

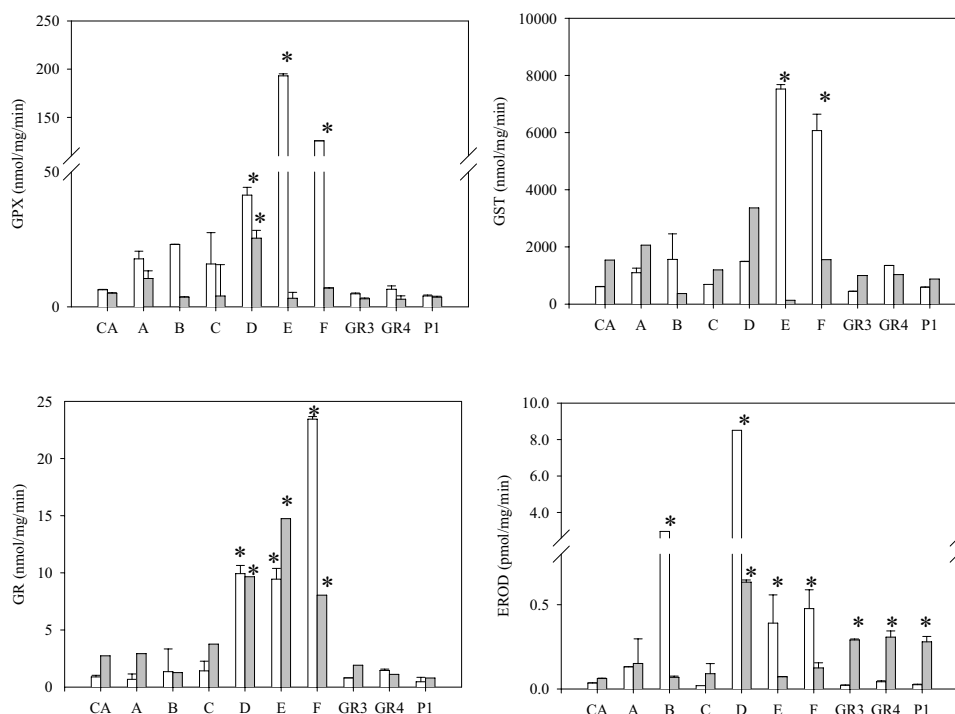
<sup>a</sup> Concentration that exceeds the ERL (Effects Range-Low) defined by NOAA (1999); <sup>b</sup> value that exceeds the sediment quality guideline suggested by DelValls&Chapman (1998); <sup>c</sup> concentration which surpass the guideline described by Riba et al. (2004); <sup>d</sup> value that exceeds the guideline proposed by McDonald et al. (1996); <sup>e</sup> concentration that surpass the guidelines defined by Dutch agencies, Tweede Kamer, vergaderjaar (1994–1995).

dry sediment) in the Bay of Algeciras; these could be considered as slightly contaminated by PAHs and adverse effects could be frequent according to McDonald et al. (1996), and in the case of GR3 the concentration of this contaminant also exceeds the guideline proposed by the Dutch agencies (Tweede Kamer, vergaderjaar, 1994-1995); on the other hand sediments from the Cies Island present the lowest concentrations of PAHs, whereas these chemicals were not detected in the sampling site located in the Bay of Cadiz. No special pattern was detected regarding to the concentration of metals in the

different sites of study; GR3 and F exceeds some international guidelines defined for the metal Zn: GR3 (MacDonald et al., 1996) and F (NOAA, 1999; DelValls and Chapman, 1998; MacDonald et al., 1996). According to McDonald et al. (1996), sediments from station D exceed the guideline proposed for Pb. Sites A and C from Cies and D, F from Corme-Laxe surpass the proposed guideline described by McDonald et al. (1996) for Cu, whereas GR3 exceeds various guidelines proposed for this metal (NOAA, 1999; MacDonald et al., 1996; Tweede Kamer, vergaderjaar, 1994-1995; Riba et al., 2004). GR3 also exceeds the guidelines for Ni proposed by different international agencies and authors (NOAA, 1999; MacDonald et al., 1996; Tweede Kamer, vergaderjaar, 1994-1995).

### 3.2. Biomarkers of exposure

Mean values of the biomarkers of exposure determined in crabs and clams obtained after the 28-d exposure are summarized in Figure 2. In general, organisms deployed in the area of Corme-Laxe (D, E, and F) present the highest induction of the biomarkers of exposure. GPX activities for crabs show significant differences ( $p < 0.01$ ) between sites D, E and F (Corme-Laxe) and the reference station CA, whereas clams exposed in site D presented also significant differences with the reference station regarding to this biomarker. Differences obtained for the phase II enzyme GST measured in crabs were significantly among D, F and the reference CA. The antioxidant enzyme GR activity for both, crabs and clams resulted significantly different from CA for the three study sites located in Corme-Laxe (D, E and F). In the case of the EROD activity which accounts for the enzymatic activity occurring in the phase I of detoxification, significant differences were detected for those crabs that were placed in the locations B (AINP), D, E and F (Corme-Laxe), and clams exposed to sediments from D (Corme-Laxe) and GR3, GR4, P1 (Bay of Algeciras).

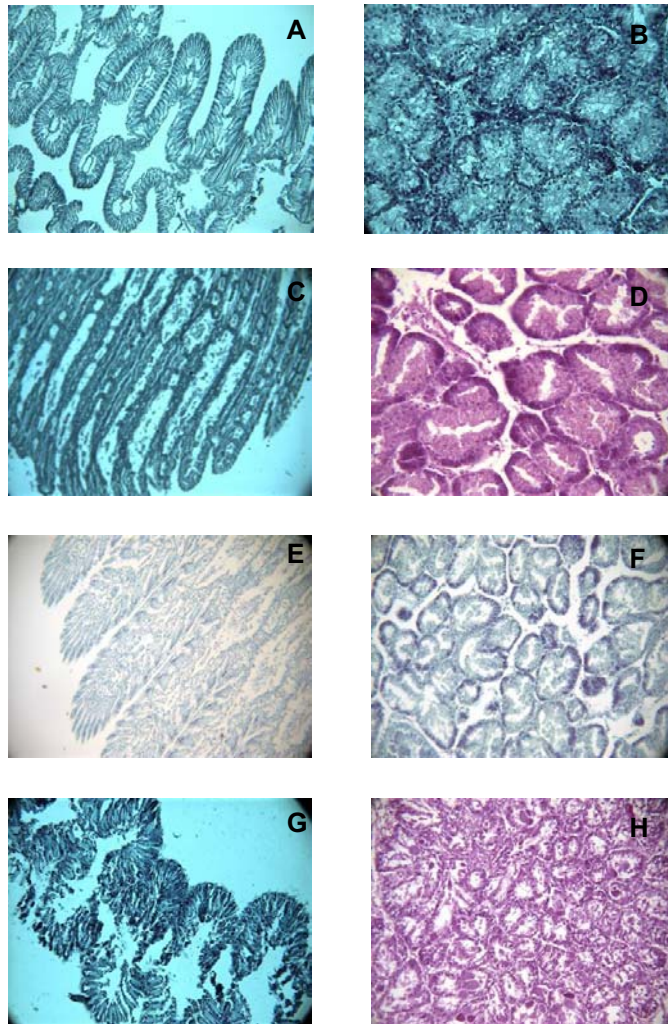


**Figure 2.** General health biomarkers for both invertebrate species, the clam *Ruditapes philippinarum* and the crab *Carcinus maenas*: glutathione peroxidase activity GPX (nmol/min/mg prot), glutathione transferase GST activity (nmol/min/mg prot), glutathione reductase GR activity (nmol/min/mg prot) and EROD activity (pmol/mg/min). Asterisks indicate significant differences with the reference treatment CA (\*p < 0.05; \*\*p < 0.01).

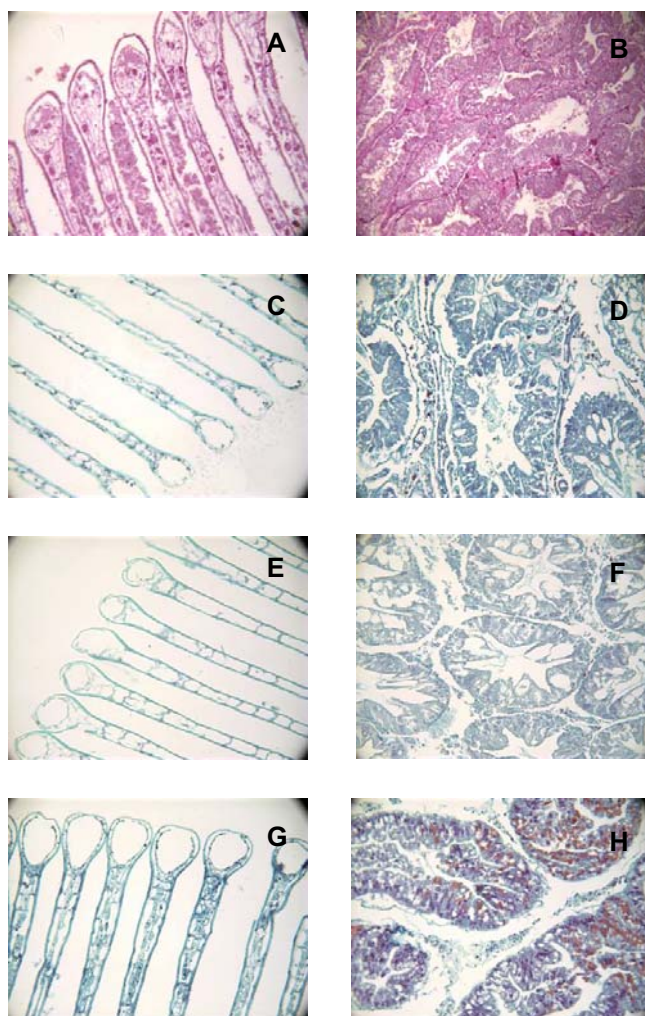
### 3.3. Biomarkers of effect

The relationship between pollutants and pathologies in target tissues has been previously reported (Ortiz-Delgado et al., 2007). Histopathology results showed no alterations in the organisms from the negative control (Figure 3 and Figure 4). In general, damage in crabs and clams tissues were lower than those detected in laboratory deployments (personal observations). Most of the

analyzed organisms showed several histological un-specific lesions related with symptoms of general stress, including loss of digestive epithelial cells, rupture of gill epithelium, respiratory lamellar fusion or lifting, as well as haemocytic infiltrates or loss of connective tissue of the gills and hepatopancreas, most which can also be observed in different marine invertebrate or vertebrate species exposed to different inorganic or organic contaminants, parasitic or infectious diseases, nutritional stress, or physico-chemical disorders (Rodriguez de la Rua et al., 2005; Ortiz-Delgado et al., 2007). Organisms exposed to sediments from the Bay of Algeciras were the most affected followed by clams and crabs exposed to sediments from Corme-Laxe, and finally organisms from the Cies treatment which showed alterations due to general environmental stress. The lesions observed in clams that had been exposed to sediments from Algeciras during 28 days included: desquamation of digestive epithelium, occlusion of the digestive ducts, haemocytic infiltrations and weak alterations or loss of the supporting digestive connective tissue (hepatopancreas), ciliar alterations, loss of support connective tissue, and hypertrophy or fusion of lamellae (gills). Crabs deployed in site GR3 presented disrupted pillar cells, epithelial changes, desquamation in gills presence of vacuoles in hepatopancreas of caged organisms. The presence of parasites in some of the crabs studied make more unclear to determine the cause of the damages, in this sense, a better relationship with pollutants was shown in clams than crabs. Organisms from the reference site did not present alterations in target tissues.



**Figure 3.** Histological sections of gills and digestive gland of the clam *Ruditapes philippinarum* after 28-d exposure to the sediments: (A) Histological section of a control gill (day 0); (B) Histological section of a control digestive gland (day 0); (C) Histological section of gill from a clam exposed to sediments from AINP; (C) Histological section of digestive gland from a clam exposed to sediments from AINP; (D) Histological section of gill from a clam exposed to sediments from Corme-Laxe; (E) Histological section of digestive gland from a clam exposed to sediments from Corme-Laxe; (F) Histological section of gill from a clam exposed to sediments from Algeciras; (G) Histological section of digestive gland from a clam exposed to sediments from Algeciras.



**Figure 4.** Histological sections of gills and hepatopancreas of the crab *Carcinus maenas* after 28-d exposure to the sediments: (A) Histological section of a control gill (day 0); (B) Histological section of a control digestive gland (day 0); (C) Histological section of gill from a clam exposed to sediments from AINP; (D) Histological section of hepatopancreas from a clam exposed to sediments from AINP; (E) Histological section of gill from a clam exposed to sediments from Corne-Laxe; (F) Histological section of hepatopancreas from a clam exposed to sediments from Corne-Laxe; (G) Histological section of gill from a clam exposed to sediments from Algeciras; (H) Histological section of hepatopancreas from a clam exposed to sediments from Algeciras.

### 3.4. Linking chemicals and biomarkers

As it has been shown above, in the current study the highest activities of biomarkers of exposure were observed in those individuals deployed “in situ” in the Bay of Corme-Laxe. Studies carried out with the same organisms and similar sediments under laboratory conditions (Morales-Caselles et al, submitted) showed higher biomarker responses in organisms exposed to sediments from the Bay of Algeciras, mainly due to the concentration of PAHs in the sediments. In some occasions in situ exposures showed greater toxicity than laboratory exposures to sediments from the same sites (Burton et al., 2005).

To elucidate the source and type of contaminant that is producing the stress to the organisms a multivariate analysis was performed to link biomarkers of exposure with the chemicals bound to sediments. Three new factors were defined to describe the 17 original variables by explaining a 75 % of the total variance (Table 2). The main Factor (29.9 %) links the phase I detoxification activity determined by EROD in clams and crabs, the GST and GPX activity in clams to the concentration of Pb in the sediment. This factor has a positive loading principally in site D from Corme-Laxe and followed by site A in Cies (Figure 5). The low score in site A suggests that Pb bound to sediments produced some stress although the high prevalence of this factor in site D, which exceeds the sediment quality guideline proposed by McDonald et al (1996) for this contaminant implies that there is a source of Pb which has involved the activation of “early warning” biomarkers. Precisely, the activation of a group of biomarkers often related to the defence against organic compounds suggests that the metal Pb comes from an organic source such as hydrocarbons.

On the other hand the fact that the biomarkers of exposure have this significant induction in the organisms exposed to sediments from the Bay of Corme-Laxe which was not detected under laboratory exposures (Morales-

Caselles et al., submitted), could be related to the existence of a non measured contaminant that might come from the sea water. In this case a possible source could be the presence of caged mussel for aquaculture in the proximities what might suppose an input of organic matter therefore a cause of stress to the deployed organisms. A source of organic matter to the surrounding water involves a decrease in the dissolved oxygen which is used in the oxidation processes. The oxygen content of water can be important in determining the nature and the rate of both chemical and biochemical transformations (Walker et al., 2006).

**Table 2.** Sorted rotated factor loadings of 17 variables for the three principal factors resulting from the multivariate analysis of results obtained from the biomarker responses in crabs and clams and the chemical analysis.

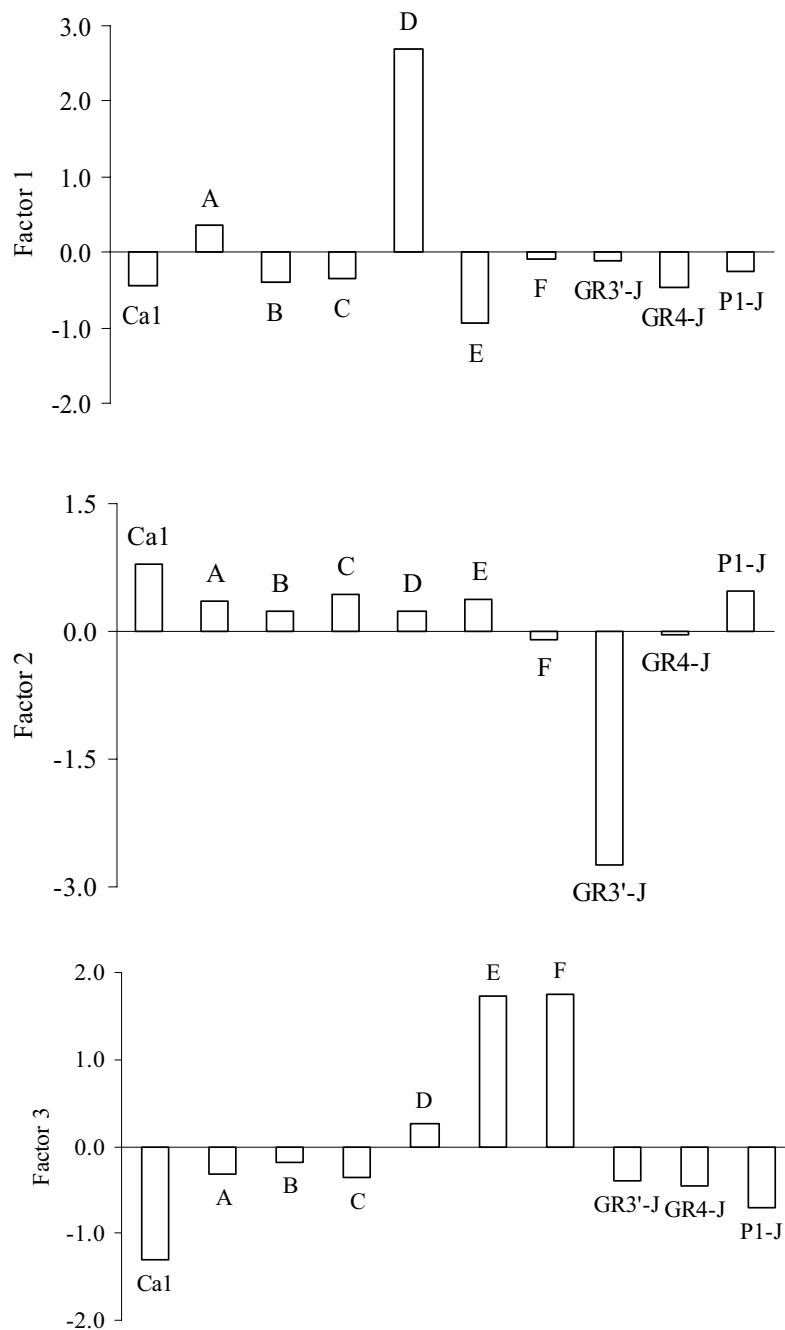
	<b>FACTOR 1</b>	<b>FACTOR 2</b>	<b>FACTOR 3</b>
	29.9	23.8	21.1
<b>GPX-crab</b>	—	—	0.93
<b>GPX-clam</b>	0.97	—	—
<b>GR-crab</b>	—	—	0.85
<b>GR-clam</b>	—	—	0.82
<b>GST-crab</b>	—	—	0.94
<b>GST-clam</b>	0.91	—	—
<b>EROD-crab</b>	0.90	—	—
<b>EROD-clam</b>	0.94	—	—
<b>PAHs</b>	—	-0.98	—
<b>PCBs</b>	—	-0.93	—
<b>Zn</b>	—	-0.46	0.51
<b>Cd</b>	—	—	—
<b>Pb</b>	0.93	—	—
<b>Cu</b>	—	—	—
<b>Ni</b>	—	-0.97	—
<b>Co</b>	—	-0.88	—
<b>V</b>	—	—	—

According to the second factor (23.8 %) a relationship is observed between the organic contaminants PAHs and PCBs with metals Ni, Co and Zn. This factor, with negative loading, does not relate the association of these contaminants with biomarkers and accounts for a contamination in sediments from sites GR3 and GR4 from the Bay of Algeciras and F in Corme-Laxe (Figure 5). Biomarker responses were expected in organisms exposed to the contamination bound to sediments from Algeciras, as it was shown in laboratory studies (Morales-Caselles et al., submitted) however biomarkers of exposure were generally low, mainly in crabs, in comparison with sediments from Corme-Laxe. The fact that the area of the deployment, in the mouth of the river Guadarranque, is submitted to the influence of natural tides could be a reason of easing the bioavailability of contaminants to the organisms. Sediments of intertidal zones along the seashore experience fluctuating oxygen levels in accordance with tidal movements (Walker et al., 2006). As the oxygen content declines, there will be a tendency for oxidative transformations to be replaced by reductive ones. Oxidations by the microsomal monooxygenase system depend upon the activation of hemoprotein molecular oxygen ( $O_2$ ) after it has been bound to an associated hemoprotein, cytochrome P450. (Walker et al., 2006).

The third factor (21.1 %) connects the concentration of Zn in sediments to the biomarkers of response related to antioxidant activity: GR induction in crabs and clams and GST and GPX activity in crabs. Sites D, E and F from the Bay of Corme-Laxe present the influence of this factor (Figure 5) what means that this area presents a stress due to the presence of pollution by Zn. Previous studies have considered the Bay of Corme-Laxe as not contaminated and have attributed the presence of metals to basal levels (Cobelo-García et al., 2005). However, regarding to site D which levels of Zn surpass several sediment quality guidelines (table 1) an anthropogenic source of this metal is probably

the cause of the stress shown in the organisms, mainly in crabs. Previous studies have shown sublethal responses in *Carcinus maenas* exposed to sediments contaminated by Zn (Martín-Díaz et al., 2005).

In general, histopathological responses have shown moderate damage in the studied organisms deployed in Corme-Laxe what suggests a successful action of the antioxidant and detoxification activities. However, defence may involve a trade-off between production and survival: increased survival may be obtained only at a cost of reduced growth or reproduction (Walker et al., 2006). In this sense, more attention should be played to those areas affected by the input of contaminants which maybe are not producing lethal responses although sublethal effects are expected and can lead to sequential changes and reach ecosystem levels.



**Figure 5.** Factor loadings for the three principal factors resulting from the multivariate analysis of results obtained from the chemical analysis and the suite of biomarkers.

## 5. Conclusions

In the present study sublethal responses have been analyzed in organisms deployed in sites affected by different spills in the area of Galicia and the gulf of Cádiz. In addition, biomarkers results have been linked with chemicals bound to sediments in order to elucidate the cause, source and bioavailability of adverse affects after exposure. A set of biomarkers including antioxidant and detoxification activities have been evaluated in addition to histopathological damages in target tissues. The use of two invertebrate species, the clam *Ruditapes philippinarum* and the crab *Carcinus maenas* with difference feeding habits provided a better assessment of the subject raised. The lowest sublethal responses were observed in organisms exposed to sediments from the Cíes Islands in the AINP, although the presence of some metals could have induced some stress in the deployed animals. This points to a recovery of the area four years after the *Prestige* oil spill although the inputs of some metals are considered a potential risk. Organisms exposed in Corme-Laxe presented high levels of stress that were not observed in laboratory exposures (Morales-Caselles et al., submitted) what suggests the impact of sources of contaminants, not only hydrocarbons, such as the material from the aquaculture cages. In the case of the bay of Algeciras the toxic effects of contaminants were probably diminish by the water removal of the tidal fluctuation although target tissues presented the highest alterations of all the study sites.

Previous studies agree with the fact that in situ caged organism approach should be used together with other assessment methods such as laboratory toxicity testing (Burton et al., 2005). In this report it has been shown how field studies have permitted to identify alternative sources of stress that are not possible to observe in laboratory experiments. Therefore, authors consider that bioassays should not limit to experimental designs under laboratory conditions and propose that field deployments provide the lacks regarding to the

uncontrolled circumstances of *in situ* surveys and the excessively control of laboratory tests.

## 6. Acknowledgments

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## Kinetic of biomarkers in the clam *Ruditapes philippinarum*

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### Abstract

The detoxification and antioxidant systems in the clam *Ruditapes philippinarum* was studied by analyzing the kinetics of a set of biomarkers. Phase I cytochrome P450A (CYP1A) levels were measured as digestive gland ethoxyresorufin O-deethylase (EROD) activity and by glutathione-S-transferase (Phase II), whereas antioxidant activity was evaluated by the glutathione reductase (GR) and glutathione peroxidase (GPX) inductions. Analyses were performed on clams exposed to PAHs contaminated sediments under field conditions after 7, 14, 21 and 28 days of exposure. Histopathological lesions were also determined to assess the effects of pollutants in target tissues such as gills and digestive glands. Bioassays were performed under field conditions using contaminated sediments from two areas of the Spanish coast affected by oil spills. Sediments from the selected sites were chemically characterized and the data obtained were correlated with the kinetic approach of biomarkers. Results show an important relationship between the phase I and II detoxification enzymes in the clam *R. philippinarum* whereas histopathological lesions were mainly related to general stress. In addition, toxicity testing following a kinetic approach under field conditions can be considered a suitable tool to monitor the pollutants impact, as well as to detect other sources of contamination.

*Keywords: oil spill, sediment quality, detoxification, histopathology, contamination*

## **1. Introduction**

Biomarkers have been shown to be useful tools in characterizing the health status of animals from impacted areas, where complex mixtures of pollutants are usually present [1, 2, 3, 4, 5]. Ecotoxicity studies based on biomarkers allow to determine the impact of environmental stressors and to easily follow the evolution of the ecosystem towards degradation or restoration [6].

Ethoxyresorufin O-deethylase activity (EROD) represents a good marker in MFO (mixed-function oxygenase), which is the first mode of detoxification of many organic pollutants (polycyclic aromatic hydrocarbons -PAHs-, polychlorinated biphenyls -PCBs-). The measurement of EROD activity is successfully used as a potential biomarker of exposure to xenobiotic contaminants in marine pollution monitoring. Glutathione-S-transferase (GST) represents a phase II detoxification enzyme but also implicated in oxidative stress events; a critical role for GSTs is obviously defence against oxidative damage and peroxidative products of DNA and lipids [7]. Due to the role that GSTs play in conjugating reactive epoxide species and other electrophiles, induction of these enzymes must be considered to be beneficial [7]. Glutathione peroxidases (GPX) catalyse mainly the reduction of organic peroxides to alcohols using reduced glutathione [8] whereas glutathione reductase (GR) is also used as antioxidant parameter. In addition, histopathological alterations in gills and digestive glands of bivalve molluscs tissues have been shown to be responsive and sensitive to a wide range of contaminants and have been developed and recommended as biomarkers for monitoring the effects of pollution [9].

The aim of this study was to assess the detoxification system of the clam *Ruditapes philippinarum* exposed to oil-contaminated sediments under field conditions by analyzing the kinetic of biomarkers of exposure related to the detoxification system and antioxidant activities.

## 2. Methodology

The study sites chosen in the present study have been affected by oil spills by a different way. The Bay of Algeciras (S Spain) suffers chronic effects due to the several spills of oil and other compounds that suppose an input of contaminants in the water and sediment of the zone. On the other hand, the Galician Coast (NW Spain) experienced one of the major accidental oil spills in Europe when in November 2002, the tanker *Prestige* started dropping heavy fuel oil beyond 66,000 tons. Three sites were selected in the Galician coast, two in the bay of Corme-Laxe (CL1, CL2) and one in the Atlantic Island National Park (AINP2); both areas were importantly affected by the *Prestige* oil spill, and have been recovered in last few years [9]. Two stations were also selected in the mouth of the river Guadarranque in the Bay of Algeciras (ALG1 and ALG2). A reference site was selected in a clean area in the Bay of Cádiz (S Spain) [4]

Individuals of *Ruditapes philippinarum* were obtained from an aquaculture farm, and after one week of acclimation, clams were deployed in cages (50cm x 25cm x 15cm) in the selected sites and sediment samples were carried to the laboratory to perform the chemical analysis. The experiments lasted 28 days and surveys were performed weekly.

The analyses of PAHs and PCBs bound to sediments were carried out according to USEPA SW-846 Method 827C78082 [11]. Briefly dried samples were Soxhlet extracted with n-hexane for 18 h, and the extracts were isolated by column chromatography on Florisile alumino-silica. PCBs and PAHs were eluted and their fractions were dried in a rotating evaporator and re-dissolved

in isooctane. Aromatic fractions were analyzed on a HewlettePackard (HP) 5890 Series II gas chromatographer coupled with an HP 5970 mass spectrometer. PAHs were analyzed by GC-MS using selected ion monitoring (SIM). Analysis of PCBs as AROCLOR 1242 and AROCLOR 1260 was performed using the same instrument with an electron capture detector (GC/ECD). For both set of organic chemicals, PAHs and AROCLOR, the analytical procedure showed agreement with the certified values of more than 90%.

Trace metal analysis were analyzed as described by Casado-Martínez et al.[12]; briefly, 2.5 g of sediments (<0.065 mm) were placed in Teflon containers and were digested in microwave (400W, 15 min, twice) with HNO<sub>3</sub> 2N. The extracts were purified by passing through a C-18 column and metals analyses were performed by anodic voltamperimetry (-Zn, Pb, Ni, Co and Cu- Metrohm Application Bulletin N° 147; - V- Metrohm Application Note N° V-81). For Hg the cold vapour technique was used and was quantified using atomic absorption spectrometry. The analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) and showed a recovery greater than 90% of the certified concentration.

Tested organisms were collected from cages and were dissected the days 0, 7, 14, 21 and 28 of exposure; the digestive gland was were extracted and kept at -80°C prior homogenization [1]. Samples were centrifuged at 10,000g for 30 min, and the supernatant was used for the detoxification activity determination and the total protein content described by Bradford [13]. Mixed function oxygenase activity, which is the first mode of detoxification of many organic pollutants, was measured using the EROD assay [14]. The oxidation of 1 mM NADPH by Glutathione Reductase activity (GR) in the presence of 10 mM oxidized glutathione was also monitored at 340 nm [15]. Glutathione Peroxidase activity (GPX) was measured according to McFarland et al. [14]. The phase II metabolizing enzyme Glutathione-S-transferase (GST) activity was

determined by monitoring the rate of conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm, methodology adapted from McFarland et al. [15]. Biomarkers results were normalized with the protein content. Correlation among chemicals and biomarkers were evaluated by the Pearson analysis ( $p < 0.05$  and  $p < 0.01$ ).

Gills and liver tissues of the clams were also fixed in phosphate buffered 10% formaldehyde (pH 7.2) for histopathology determination after 28 days of exposure. After dehydration in graded concentrations of ethanol, the samples were embedded in paraffin wax. Histological sections of 6 to 8  $\mu\text{m}$  thickness were stained with Haematoxylin–Eosin and Haematoxylin–VOF [16]. Sections were reviewed by light microscopy Leitz Laborlux S and photographed (Sony DKC-CM30).

### **3. Results**

#### **3.1. Chemical analysis**

Chemical analysis results are shown in table 1. The concentration of PAHs in sediments is higher in the sediments from the Bay of Algeciras located in the site ALG1 ( $2961 \mu\text{g kg}^{-1}$  dry weight), followed by those from CL1 ( $820 \mu\text{g kg}^{-1}$  dry weight) in Corme-Laxe and ALG2 ( $802 \mu\text{g kg}^{-1}$  dry weight) also in Algeciras. Sediments from the AINP present the lowest PAHs content in their sediments. ALG2 also presented the highest concentration of PCBs ( $22 \text{ mg kg}^{-1}$  dry weight), Cd ( $0.17 \text{ mg kg}^{-1}$  dry weight), Ni ( $74.7 \text{ mg kg}^{-1}$  dry weight), Co ( $12.8 \text{ mg kg}^{-1}$  dry weight) and V ( $26.1 \text{ mg kg}^{-1}$  dry weight). The major amount of Zn was found in sediments from CL1 ( $244 \text{ mg kg}^{-1}$  dry weight), whereas the highest concentration of Pb was analyzed in sediments from CL2 ( $44 \text{ mg kg}^{-1}$  dry weight) both sites in Corme-Laxe. Cu was higher in AINP2 ( $31.6 \text{ mg kg}^{-1}$  dry weight). Organic contamination was not detected in

the reference site (CA) whereas the metal content in sediments was relatively low.

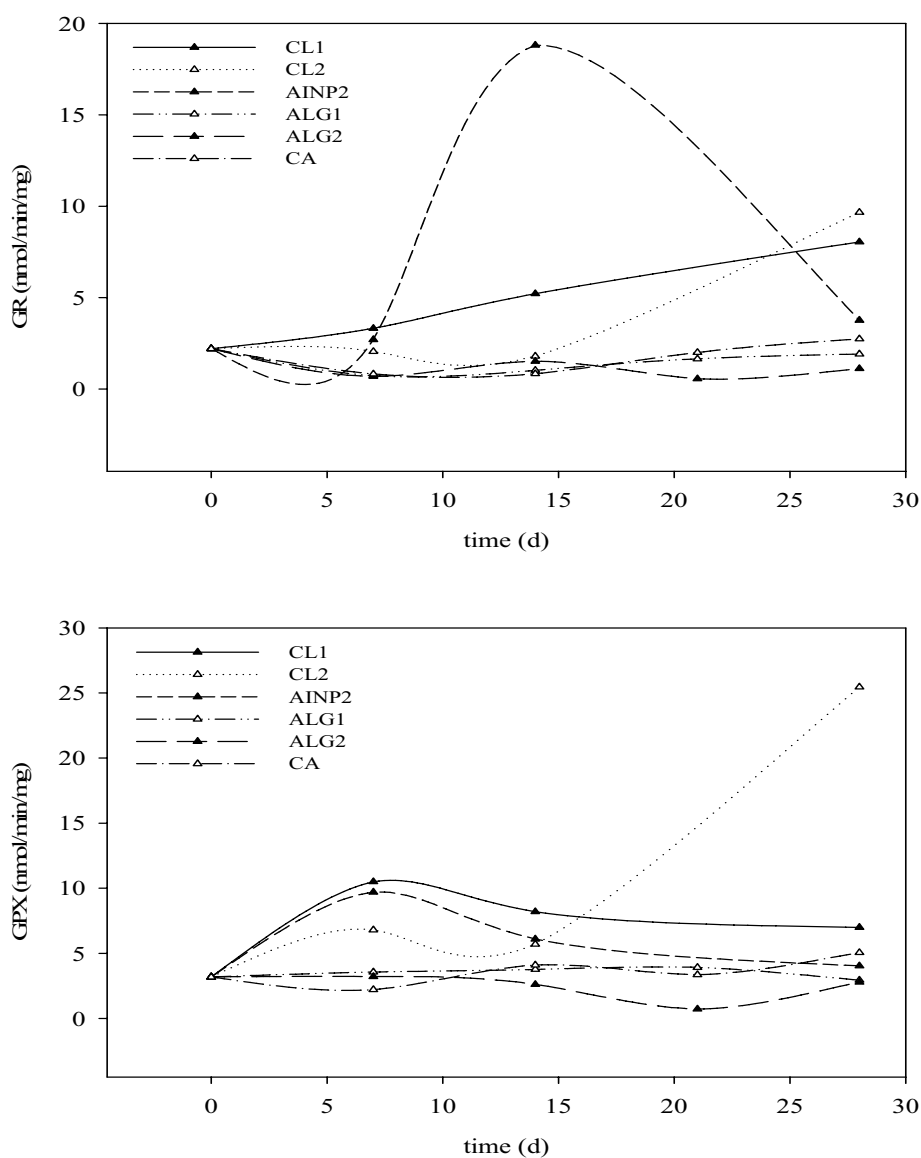
**Table 1.** Concentration of PAHs and PCBs ( $\mu\text{g kg}^{-1}$  dry weight) and metals ( $\text{mg kg}^{-1}$  dry weight) in the sediments collected from the study sites (AINP: Atlantic National Park, Galicia; CL: Corme-Laxe, Galicia; ALG: Bay of Algeciras; CA: Bay of Cádiz). n.d.: not detected.

	PAHs	PCBs	Zn	Pb	Cu	Ni	Co
<b>AINP2</b>	239	4.76	37.5	6.54	31.6	5.02	0.87
<b>CL1</b>	820	2.28	244	14.3	19.1	7.03	0.67
<b>CL2</b>	537	2.60	65.7	44.0	22.1	9.39	1.21
<b>ALG1</b>	2961	22.0	138	21.6	5.01	74.7	12.8
<b>ALG2</b>	802	1.75	35.3	6.21	3.67	13.1	5.59
<b>CA</b>	n.d	n.d.	21.3	2.28	6.98	0.06	3.40

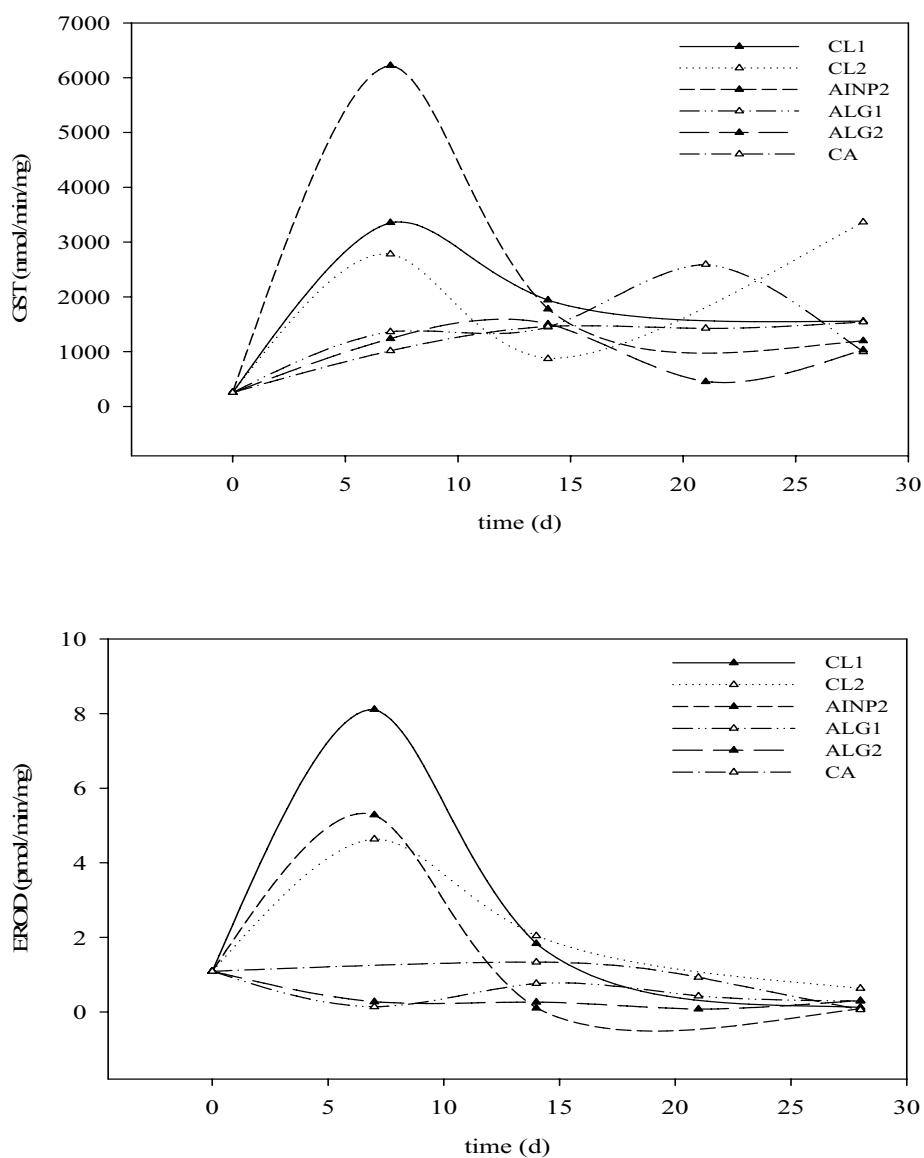
### 3.2. Biomarkers of exposure

Biomarker responses of deployed animals are summarized in Figure 1 and Figure 2. Clams deployed in sites located in the Galician Coast presented an initial increase of GPX induction during the first week of exposure (significantly different to the reference CA,  $p < 0.01$ ) while the following weeks the activity reached basal levels except for site CL2 which continued rising showing a maximum after 28 days (significantly different to the reference CA,  $p < 0.01$ ). The behaviour of this biomarker in organisms collected from cages placed in the Bay of Algeciras was similar to those from the reference site, although differences were significant ( $p < 0.01$ ). The GR activity showed significant ( $p < 0.01$ ) differences to the reference CA for the samples collected in the Galician Coast; this biomarker presented an increase along the exposure period in clams from Corme-Laxe whereas the other study sites presents similar

induction as the reference site, except for a peak detected in the clams from the AINP which appeared after 14 days of deployment (significantly different to the reference CA,  $p < 0.01$ ). ALG2 also showed significant differences ( $p < 0.05$ ) to CA the last day of survey. During the first week of experiment the GST activity increased especially in clams collected in the Galician Coast (significantly different to the reference CA,  $p < 0.01$ ); a second maximum was observed in the last survey in site CL2. A peak was detected after 21 days in clams collected from ALG1 in the Bay of Algeciras. EROD activity also presented an initial induction during the first week of exposure for organisms deployed in sites from the Galician Coast although the general pattern was to stabilize along the time, showing similar results the last day of the experiment. Significant differences ( $p < 0.01$ ) with the reference site CA were observed for all study sites.



**Figure 1.** GPX and GR activities (nmol/min/mg protein) measured in the digestive gland of *R. philippinarum* exposed along 28 days to oil contaminated sediments (AINP: Atlantic National Park, Galicia; CL: Corme-Laxe, Galicia; ALG: Bay of Algeciras) under field conditions



**Figure 2.** GST (nmol/min/mg protein) and EROD (pmol/mg/min) activities in the digestive gland of *R. philippinarum* exposed along 28 days to oil contaminated sediments (AINP: Atlantic National Park, Galicia; CL: Corme-Laxe, Galicia; ALG: Bay of Algeiras) under field conditions.

### 3.3. Biomarkers of effect

After 28 days of exposure to the sediments clams presented different alterations in target tissues (gills and gut). Most of the analyzed organisms show lesions related with general stress, including loss of epithelial cells, lamellae separation and haematitic reaction. Organisms exposed to sediments from the Bay of Algeciras were the most affected followed by clams exposed to sediments from Corne-Laxe, and finally clams from the Cies treatment which showed alterations due to general environmental stress.

## 4. Discussion

The fluctuation of different biomarkers in response to different toxicants provides a pattern of results which can give clues as to the type of pollutant that is causing the observed effect [17]. In general, results obtained in this study showed an induction of GPX, GST and EROD activity presented a maximum after the first 7 days of exposure, whereas GR shows an increase along the deployment period. Correlations among biomarkers (table 2) were significant for the induction of the detoxification system determined by EROD activity and the antioxidant enzymes GR and GPX the day 7 of exposure. This points to the fact that organisms responded to environmental stressors mainly during the first days of deployment. The correlations observed indicated a relationship among organic contaminants (PAHs and PCBs) and metals Ni and Co in the sediments, although no links were detected with biomarkers. A connection was detected between Pb and the induction of detoxification and antioxidant enzymes (GPX, GST and EROD activities) after 28 days of exposure. The Pb origin in polluting oil was corroborated by other authors [18]; there is often a strong relationship between lead concentrations in soil and parent material [19], so this compound could be related to an organic contamination associated with a source of hydrocarbons not linked to the PAHs bound sediment, and which is

**Table 2.** Pearson correlation (\*p<0.05, \*\*p<0.01) results among chemical compounds bound to sediments and biomarkers: glutathione peroxidase (GPX) activity, glutathione reductase (GR), glutathione-S-transferase (GST) activity and Ethoxyresorufin O-deethylase (EROD) activity. 7, 14, 21 and 28 correspond to the sampling date

	PAHs	PCBs	Zn	Pb	Cu	Ni	Co	GPX	GPX	GPX	GR	GR	GST	GST	GST	EROD	EROD	EROD	EROD
	1	0.905 <sup>†</sup>	0.458	0.214	-0.149	0.969 <sup>†</sup>	0.863 <sup>†</sup>	-0.209	-0.245	0.299	-0.185	-0.345	-0.269	-0.042	-0.133	-0.208	-0.117	0.758	-0.144
PCBs	0.905 <sup>†</sup>	1	0.275	0.118	-0.297	0.914 <sup>†</sup>	0.799 <sup>†</sup>	-0.109	-0.002	0.411	-0.238	-0.192	-0.044	0.129	-0.124	0.016	0.079	0.869	-0.255
Zn	0.458	0.275	1	0.266	0.020	0.335	0.187	0.492	0.294	0.485	0.068	0.414	-0.035	0.125	0.128	0.094	0.148	0.817	0.17
Pb	0.214	0.118	0.266	1	0.129	0.260	0.059	0.213	0.059	0.475	0.874 <sup>†</sup>	0.171	-0.203	0.061	0.172	-0.078	-0.640	0.702	0.811 <sup>†</sup>
Cu	-0.149	-0.297	0.020	0.129	1	-0.123	-0.342	-0.052	-0.299	0.31	0.076	-0.131	0.138	0.121	-0.236	0.053	-0.522	-0.293	0.143
Ni	0.969 <sup>†</sup>	0.914 <sup>†</sup>	0.335	0.260	-0.123	1	0.882 <sup>†</sup>	-0.279	-0.277	0.383	-0.166	-0.406	-0.239	0.056	-0.258	-0.261	-0.185	0.817	-0.095
Co	0.863 <sup>†</sup>	0.799 <sup>†</sup>	0.187	0.059	-0.342	0.882 <sup>†</sup>	1	-0.431	-0.423	0.114	-0.231	-0.566	-0.274	-0.044	-0.312	-0.307	-0.182	0.746	-0.073
GPX 7	-0.209	-0.109	0.492	0.213	-0.052	0.882 <sup>†</sup>	-0.431	1	0.675	-0.212	0.27	0.949 <sup>†</sup>	0.699	-0.482	0.628	0.778 <sup>‡</sup>	0.483	0.416	0.26
GPX 14	-0.245	-0.002	0.294	0.059	-0.299	-0.277	-0.423	0.675	1	0.581	0.124	0.86 <sup>†</sup>	0.321	0.767	0.137	0.496	0.559	0.738	-0.029
GPX 21	0.299	0.411	0.485	0.475	0.310	0.383	0.114	-0.212	0.581	1	0.388	-0.028	-0.907	0.894	0.392	-0.176	-0.40	0.743	0.105
GPX 28	-0.185	-0.238	0.068	0.874 <sup>†</sup>	0.076	-0.166	-0.231	0.27	0.124	0.388	1	0.274	-0.115	0.744	0.366	0.067	-0.583	-0.013	0.906 <sup>†</sup>
GR 7	-0.345	-0.192	0.414	0.171	-0.131	-0.406	-0.566	0.949 <sup>†</sup>	0.860 <sup>†</sup>	-0.028	0.274	1	0.586	0.345	0.579	0.691 <sup>‡</sup>	0.568	0.221	0.199
GR 14	-0.269	-0.044	-0.035	-0.203	0.138	-0.239	-0.274	0.699	0.321	-0.907	-0.115	0.586	1	-0.926	-0.003	0.947 <sup>†</sup>	0.347	-0.417	-0.035
GR 21	-0.042	0.129	0.125	0.061	0.121	0.056	-0.044	-0.482	0.767	0.894	0.744	0.345	-0.926	1	0.685	-0.389	-0.192	0.595	0.529
GR 28	-0.133	-0.124	0.128	0.172	-0.236	-0.258	-0.312	0.628	0.137	0.392	0.366	0.579	-0.003	0.685	1	0.403	0.223	0.528	0.114
GST 7	-0.208	0.016	0.094	-0.078	0.053	-0.261	-0.307	0.496	0.137	0.392	0.067	0.691 <sup>‡</sup>	0.947 <sup>†</sup>	-0.389	0.403	1	0.412	0.495	0.035
GST 14	-0.117	0.079	0.148	-0.640	-0.522	-0.185	-0.182	0.483	0.559	-0.400	-0.583	0.568	0.347	-0.192	0.223	0.412	1	0.193	-0.679 <sup>‡</sup>
GST 21	0.758	0.869	0.817	0.702	-0.293	0.817	0.746	0.416	0.738	0.743	-0.013	0.221	-0.417	0.595	0.528	0.495	0.193	1	0.077
GST 28	-0.144	-0.255	0.170	0.811 <sup>†</sup>	0.143	-0.095	-0.073	0.26	-0.029	0.105	0.906 <sup>†</sup>	0.199	-0.035	0.529	0.114	0.035	-0.679 <sup>‡</sup>	0.077	1
EROD 7	-0.479	-0.317	0.421	0.085	-0.190	-0.548	-0.583	0.934 <sup>†</sup>	0.837 <sup>‡</sup>	-0.858	0.305	0.988 <sup>†</sup>	0.473	-0.846	0.662	0.638	0.523	-0.984	0.27
EROD 14	-0.208	0.025	-0.024	0.047	-0.421	-0.206	-0.323	0.176	0.822 <sup>†</sup>	0.455	0.095	0.459	-0.146	0.757	0.06	0.014	0.416	0.5	-0.166
EROD 21	-0.313	-0.117	-0.198	-0.304	-0.100	-0.22	-0.144	-0.611	0.816	0.641	0.919	0.635	-0.768	0.917	0.843	-0.479	0.056	0.382	0.818
EROD 28	0.272	0.088	0.041	0.793 <sup>†</sup>	0.225	0.257	0.215	-0.152	-0.310	-0.321	0.740 <sup>†</sup>	-0.242	-0.323	-0.712	0.081	-0.158	-0.824 <sup>‡</sup>	-0.087	0.677 <sup>‡</sup>

producing the activation of these defence systems. Lead is not essential to metabolism and it is highly toxic for biota, this toxicity is determined by their ability to regulate anomalous concentrations, through various detoxification mechanisms [20]. The correlation shown between Pb and biomarkers occurs in the day 28 what suggests that it is after this period when the metal became bioreactive to the studied biomarkers.

In general, the behaviour of GST was similar to EROD in most of the cases what suggests a relationship among these phase I and II detoxification biomarkers. The first increase of the biomarkers of effect denotes an initial activation of the detoxification system which is inhibited in subsequently surveys. When there is an increase on the CYP 450 content, there is necessarily an increment of metabolites to be conjugated with phase II enzymes, thus preventing cell damage; when these water soluble compounds are conjugated with phase II enzymes, GST may intervene [21].

Previous studies with other organisms have observed both increase and decrease of GPX activity in field surveys [7]; results obtained in this investigation show an initial fluctuation of this biomarker (increase in the Galician Coast and decrease in the Bay of Algeciras) followed by a estabilization except for site CL2 in Corme-Laxe.

The activation of GR plays a fundamental role in the face of oxidative stress maintaining the proper redox status of glutathione, which is important both as cofactor of several antioxidant enzymes and as indirect scavenger of oxyradicals [22]. In the present study organisms exposed to sediments from Corme-Laxe present a continuous induction of this biomarker suggesting the presence of a chronic source of stress which is not related to the contaminants measured in the sediments, but probably related to the stress produced by the pressure of the mussel rafts [23] which may produce negative impacts to the organisms exposed.

Biomarker fluctuations were relatively low in site CA in comparison with the other study sites, what confirms the feasibility of this location as reference site in ecotoxicological studies. Higher biomarker responses were expected in organisms exposed to the contamination bound to sediments from the Bay of Algeciras, as it was shown in laboratory studies [24] however biomarkers of exposure were generally low in comparison with organisms exposed to sediments from Corme-Laxe. In the case of the caged organisms located in the mouth of the river Guadarranque the variations of abiotic factors due to the influence of tides could affect the activity of the studied biomarkers. Previous studies have demonstrated the fluctuations of detoxification enzymes in response to changes of temperature and salinity [21].

The histopathological symptoms of stress agree with the presence of contaminants not only in the sediment but also in the water in the areas of Corme-Laxe and Algeciras. Organisms deployed in the AINP presented slight reversible lesions related with general stress but not with contaminants. The lesions were similar to the tissues from organisms exposed to sediments from the reference station in the Bay of Cádiz. On the whole, organisms' laboratory deployments (personal observations) have concurred in more incidences of lesions in target tissues than in the field exposures what suggests that the pollutants come mainly from the sediments; under field conditions the effects of the contaminants bound to sediment are relieved due to the open water system what diminish bioavailability of contaminants. The application of biomarkers under field conditions involves more realistic conditions for the experiment [25, 26, 27], however a lot of uncontrolled variables may affect biomarkers; previous studies agree with the fact that in situ caged organism approach should be used in tandem with other assessment methods such as laboratory toxicity testing [28].

## 5. Conclusions

In the present study a set of biomarkers involved in the detoxification system, antioxidant activities and one biomarker of effect (histopathology) were assessed in the clam *Ruditapes philippinarum* exposed under field conditions to sediment samples affected by oil spills. The set of the studied biomarkers presented an important induction during the first week of deployment and a connection of the phase I and II enzymatic activities of the detoxification system in the clam *Ruditapes philippinarum* was suggested. The toxicity of Pb bound to sediments was related to the induction of biomarkers after 28 days of deployment, what indicates the importance of carrying out kinetic studies in field studies where the bioavailability of contaminants partially depends on abiotic parameters. In addition the evaluation of biomarkers along the time has allowed to distinguish different sources of contaminants not related to sediments.

Authors consider that *Ruditapes philippinarum* is a suitable species in oil contaminated sediments assessment by including a set of antioxidant, phase I and II detoxification biomarkers together with biomarkers of effect such as histopathology; moreover toxicity testing following a kinetic approach under field conditions contributes in a very effective way to monitor and determine the pollutants effects, including those that have not been analyzed.

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## **A kinetic approach in the PAHs detoxification system in a marine invertebrate specie: the crab *Carcinus maenas***

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### **Abstract**

To test the hypothesis that invertebrates present a significant PAHs-detoxification system, the induction of ethoxyresorufin O-deethylase (EROD) activity and guthatione-S-transferase (GST) was studied in the crab *Carcinus maenas*. A bioassay was performed by exposing the organisms to bulk sediment contaminated by PAHs under laboratory and field conditions. Sediments were collected and transferred to the laboratory where they were subsampled for chemical analysis and toxicity test; crabs were kept during 28 days in tanks with the collected sediment samples whereas caged crabs were also placed in the selected study sites during the same period. Sampling was performed weekly and hepatothopancreas were homogenized and centrifuged for the biomarkers determination. Results obtained show the relationship between the kinetic of the biomarkers measured in the crabs and the chemical characteristics of the sediment. Besides, it demonstrates the capability of the mentioned biological systems involved in the detoxification of PAHs toxicity in the studied organism.

*Keywords: biomarker, invertebrate, histopathology, EROD activity, GST.*

## 1. Introduction

The presence of a xenobiotic compound in a segment of an aquatic ecosystem does not, by itself, indicate injurious effects. Connections must be established between external levels of exposure, internal levels of tissue contamination and early adverse effects (Van der Oost, 2003). Sediment-associated chemicals may or may not be bioavailable, and there is a paucity of information on their combined effects on exposed organisms (Werner et al., 2004). A variety of molecular, biochemical, physiological, histopathological, organisimal, population and community responses may be used to identify exposure to certain chemicals, provide information on spatial and temporal changes in the concentration of contaminants, and indicate environmental quality or occurrence of adverse ecological consequences. (Au, 2004). The use of biomarkers which are indicative of PAHs exposure may provide an early warning of potential ecosystem degradation, contaminant bioavailability, and the defence responses of exposed organisms (Goksøyr et al., 1996; Goksøyr & Förlin, 1992; Reynolds et al., 2003).

The EROD activity is used as a biomarker of exposure to lipophilic organic contaminants such as PAHs and measures the enzymatic activity of the phase I catalyzed by the complex CYP1A which transforms some lipophilic xenobiotics in metabolites more water soluble (Bach et al., 2005). Glutathione-S-transferase (GST) represents a phase II detoxification enzyme but also implicated in oxidative stress events; a critical role for GSTs is obviously defence against oxidative damage and peroxidative products of DNA and lipids (Van der Oost, 2003). On the other hand the capacity of many pollutants to alter different cells, tissues or organs has led to design histopathological techniques in order to evaluate the effects of contaminants (Lowe, 1988; Sarasquete et al., 1997).

In the present study bioassays under field and laboratory conditions have been developed to elucidate the detoxification system in the crab *Carcinus maenas* exposed to sediments affected by oil spills, by analyzing the kinetic of two biomarkers of exposure related to the detoxification system (EROD and GST activities) and one biomarker of effect (histopathology).

## **2. Material and methods**

### **2.1. Study sites**

Three different areas were selected to carry out the bioassays, two of them in the Galician Coast (NW Spain) (the Bay of Corme-Laxe and the Cies Island) and the other one in the Bay of Algeciras (S Spain). In total 9 study sites were chosen, 3 in the Cies Islands (A, B, and C), 3 in the Bay of Corme-Laxe (D, E and F) and 3 in the Bay of Algeciras (GR3, GR4 and P1). The area of Galicia was affected in 2002 by the spill of the tanker *Prestige* whereas the Bay of Algeciras suffers continuous spills of oil and other compounds from the industries and the maritime activities of the area.

### **2.2. Chemicals in sediments**

For PAHs and PCBs determination dried samples were Soxhlet extracted with n-hexane for 18 h, and the extracts were isolated by column chromatography on Florisile alumino-silica. PCBs and PAHs were eluted and their fractions were dried in a rotating evaporator and re-dissolved in isooctane. PAHs were analyzed by GC-MS using selected ion monitoring (SIM). Analysis of PCBs as AROCLOR 1242 and AROCLOR 1260 was performed using the same instrument with an electron capture detector (GC/ECD). Trace metal analysis were analyzed as described by Casado-Martínez et al. (2006); sediment samples were digested in microwave (400W, 15 min, twice) with HNO<sub>3</sub> 2N, the extracts were purified by passing through a C-18 column and metals analyses

were performed by anodic voltamperimetry (-Zn, Cd, Pb, Ni, Co and Cu- Metrohm Application Bulletin N° 147; - V- Metrohm Application Note N° V-81). For Hg the cold vapour technique was used and was quantified using atomic absorption spectrometry.

### 2.3. Field and laboratory bioassays

Intermoult female *Carcinus maenas* were collected from a clean site of the Bay of Cádiz (Riba et al., 2004) from an aquaculture farm and were kept under laboratory conditions during three weeks for acclimatization. After that period the crabs were placed in cages which were deployed in the study sites to conduct the field bioassay; simultaneously, about 4 L of sediment from the reference site and the other stations were placed in replicate 25-L glass tanks with clean sea water before the beginning of the experiment. After particle settling, aeration was provided to maintain adequate oxygen concentrations (greater than 80% saturation). Crabs were also located in these tanks containing sediment from the different stations in order to perform the toxicity test under laboratory conditions. Both bioassays under laboratory and field conditions were carried out during 28 days and over this time crabs were fed every week with a mixed diet of mussels or fish whereas water from the laboratory tanks was replaced every three days.

### 2.4. Biochemical analysis

Sampling was conducted every week during the 28 days of the exposure period; after dissection, hepatopancreas was kept at -80°C prior homogenization. The samples were homogenized following the procedure developed by Lafontaine et al. (2000). Mixed function oxygenase activity, implicated in monooxygenation reactions of dioxins and PAHs, was measured using the adapted Ethoxyresorufin O-deethylase (EROD) (Gagnè and Blaise, 1993). The phase II metabolizing enzyme Glutathione-S-transferase (GST)

activity was determined by monitoring the rate of conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (McFarland et al., 1999).

Gills and hepatopancreas tissues were fixed in phosphate buffered 10% formaldehyde (pH 7.2) for histopathology determination after 28 days of exposure. After dehydration in graded concentrations of ethanol, the samples were embedded in paraffin wax. Histological sections of 6 to 8  $\mu\text{m}$  thickness were stained with Haematoxylin–Eosin and Haematoxylin–VOF (Gutiérrez, 1967). Sections were reviewed by light microscopy Leitz Laborlux S and photographed (Sony DKC-CM30).

### 3. Results

#### 3.1. Chemical analysis

Sediments from the Cies Island presented the lowest concentrations of PAHs, whereas the highest concentration of PAHs was found in the sediments from GR3 (2961  $\text{mg Kg}^{-1}$  dry sediment) located in the Bay of Algeciras, followed by sediments from the station F (820  $\text{mg Kg}^{-1}$  dry sediment) located in Corme-Laxe, GR4 (802  $\text{mg Kg}^{-1}$  dry sediment) and P1 (641  $\text{mg Kg}^{-1}$  dry sediment) in the Bay of Algeciras. High concentrations were detected for: Zn in GR3 (138  $\text{mg Kg}^{-1}$  dry sediment) and F (243  $\text{mg Kg}^{-1}$  dry sediment); Pb in site D (44  $\text{mg Kg}^{-1}$  dry sediment); Cu: A (18.9  $\text{mg Kg}^{-1}$  dry sediment ) and C (31.6  $\text{mg Kg}^{-1}$  dry sediment ) from Cies and D (22.1  $\text{mg Kg}^{-1}$  dry sediment), F (19.1  $\text{mg Kg}^{-1}$  dry sediment) from Corme-Laxe and P1 (75.2  $\text{mg Kg}^{-1}$  dry sediment) from the Bay of Algeciras; Ni in site GR3 (74.7  $\text{mg Kg}^{-1}$  dry sediment).

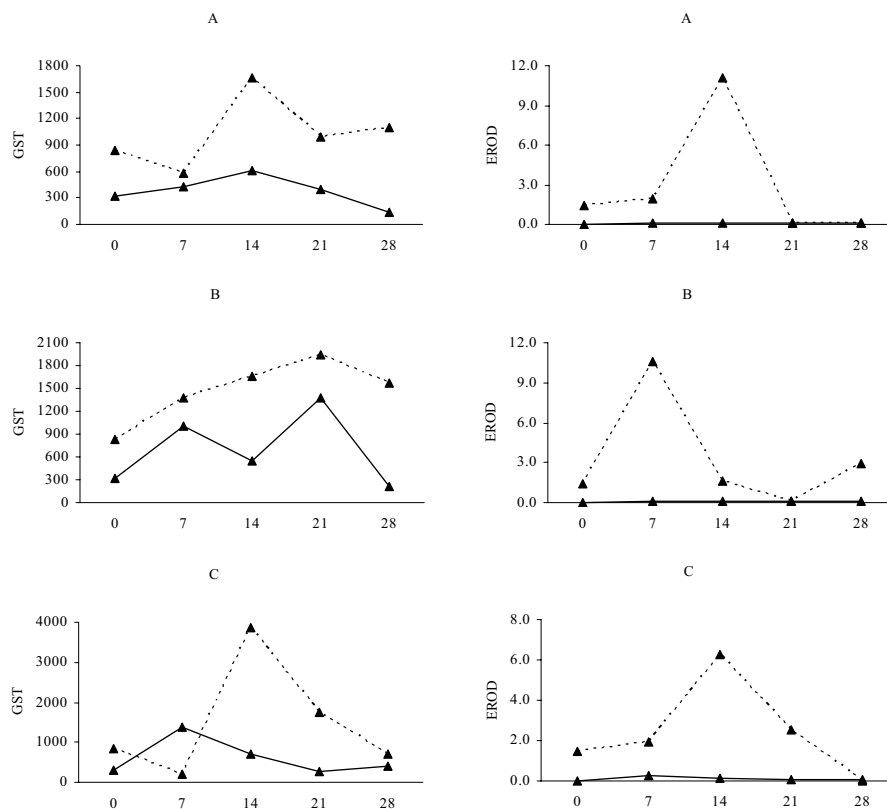
#### 3.2. GST and EROD activities

Analysis performed in crabs exposed to sediments from the Cies Islands showed that during 28 days of exposure EROD activity under field conditions

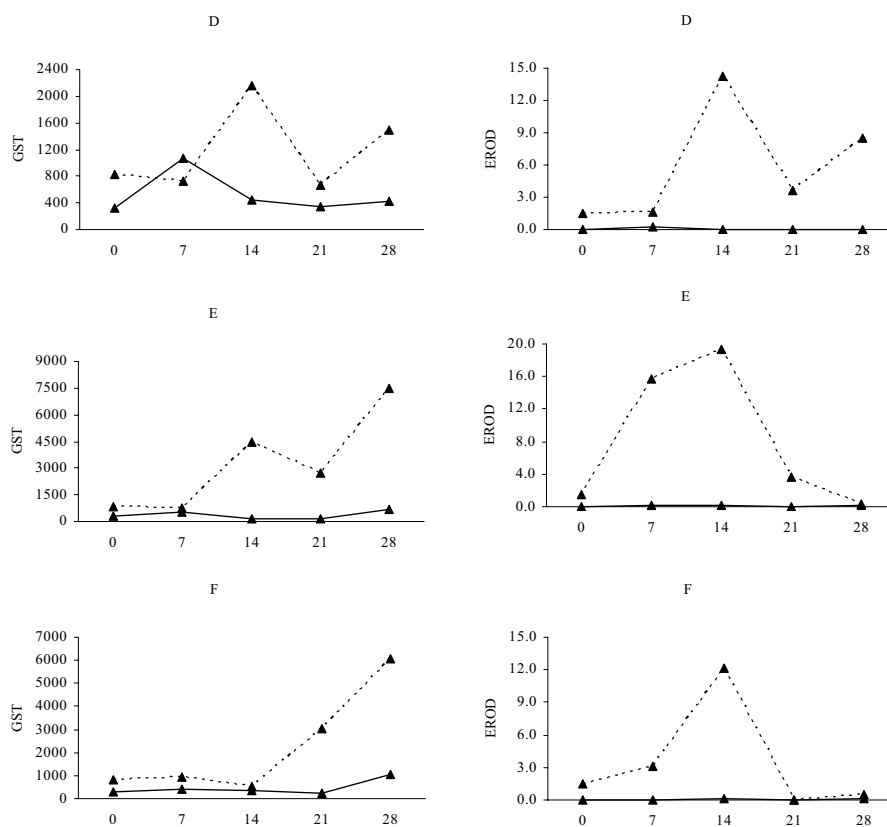
kept low and no significant differences were detected in relation with the day 0. Under field conditions crabs deployed in sites A, B and C presented a peak in the induction of this system after 7 -14 days of the beginning of the bioassay; the activity of the GST enzyme was also higher in crabs exposed under field conditions, and the maximum was observed after 14 – 21 days of exposure.

The enzymes activities detected in crabs exposed to sediments from the Bay of Corme-Laxe was higher for those organisms deployed in field than crabs from the laboratory assays. The induction of EROD and GST activities under controlled conditions in laboratory was not significant compared with the field deployments where EROD activity showed a maximum the day 14 of exposure in crabs from sites D, E and F. GST activity analyzed in crabs from cages located in station D presented similar behaviour than EROD activity, with a peak of induction the day 14. In contrast, crabs collected in sites E and F showed a maximum of induction the day 28 of exposure, which correspond to the last day of the experiment.

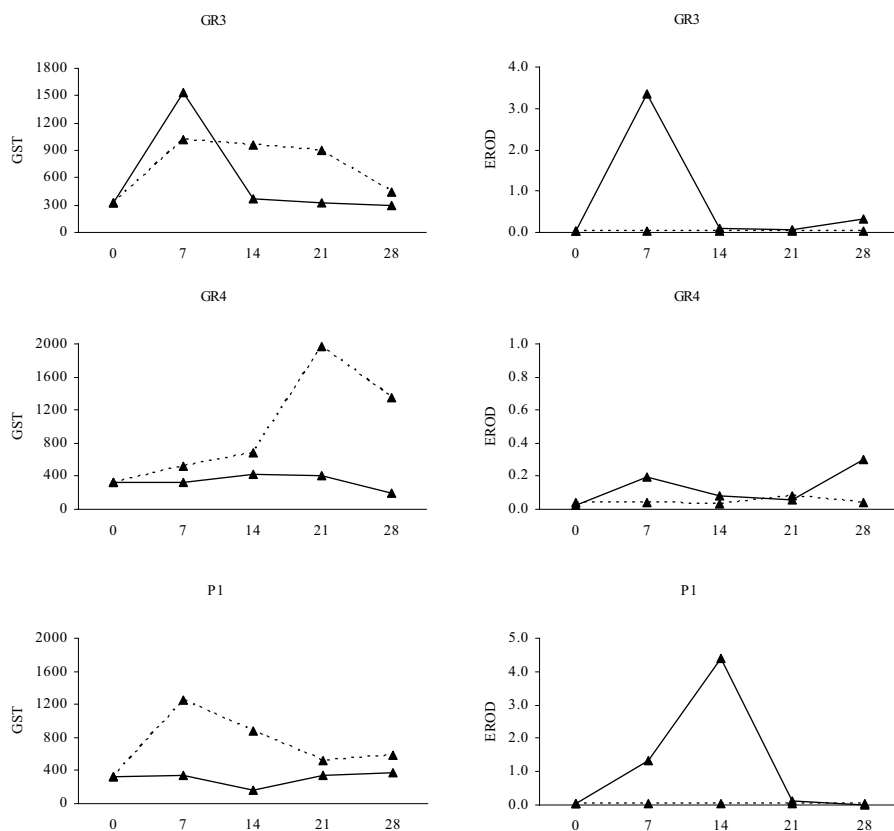
Crabs exposed to sediments from the Bay of Algeciras presented the highest values of EROD activity in laboratory exposures. In the case of site GR3 which is the most contaminated site by PAHs, the maximum induction was observed a week after the beginning of the assay in crabs exposed under laboratory conditions; a similar behaviour was followed by the induction of GST activity analyzed in the same crabs. A maximum on of EROD activity was also observed the day 14 in crabs exposed in laboratory to sediments collected from site P1. Organisms from GR4 did not present significant inductions of EROD activity under field and laboratory assays in comparison with other sites. In general, under field deployments the induction of GST was higher than in crabs from laboratory assays. A peak in GST activity was observed the day 21 in organisms from GR4 and day 7 for crabs caged in site P1.



**Figure 1.** GST (nmol/min/mg protein) and EROD (pmol/mg/min) activities in the hepatopancreas of *Carcinus maenas* exposed along 28 days to sediments from the Cies Islands. Straight line: laboratory assays; dotted line: field assays.



**Figure 2.** GST (nmol/min/mg protein) and EROD (pmol/mg/min) activities in the hepatopancreas of *Carcinus maenas* exposed along 28 days to sediments from the Bay of Corne-Laxe. Straight line: laboratory assays; dotted line: field assays.



**Figure 3.** GST (nmol/min/mg protein) and EROD (pmol/mg/min) activities in the hepatopancreas of *Carcinus maenas* exposed along 28 days to sediments from the Bay of Algeciras. Straight line: laboratory assays; dotted line: field assays.

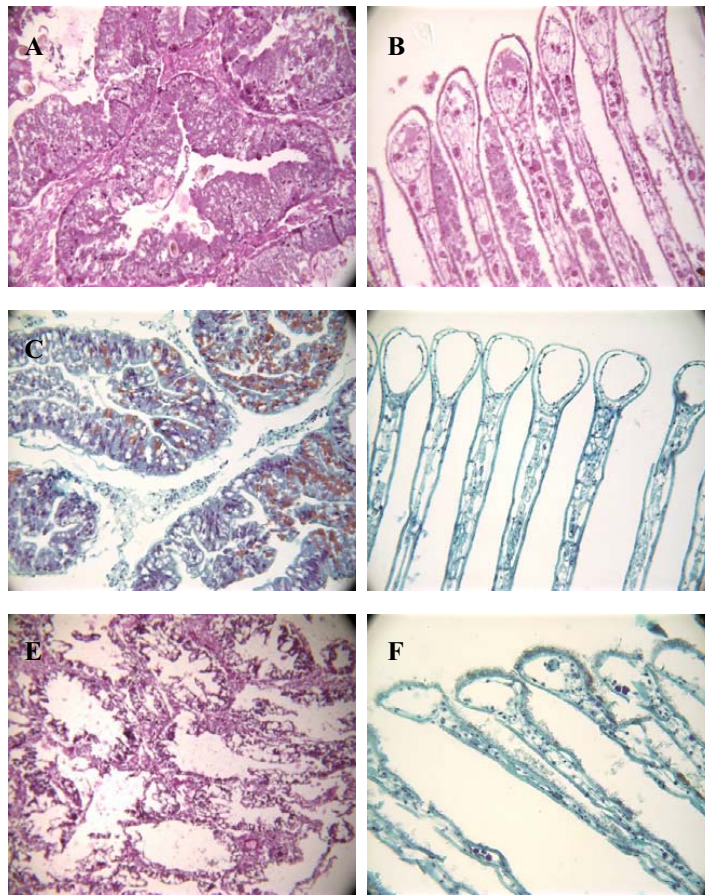
### 3.3. Histological lesions

As desirable no alterations were detected the day 0 of exposure prior to the beginning of the tests. All deployed organisms presented alterations related with general stress, such as loss of connective tissue of the gills and hepatopancreas, rupture of gill epithelium, and haemocytic infiltrates. In general, damage was higher in sites from Algeciras followed by Corme-Laxe and then the Cies Islands which tissues were similar to controls. Alterations in crabs were lower under field exposures than those detected in laboratory deployments; no important lesions were detected except for site GR3 where different alterations were observed, including: disrupted pillar cells, epithelial changes, desquamation in gills, presence of vacuoles in hepatopancreas, loss of support connective tissue, necrosis, etc.

### 3.4. Correlation among chemicals and biomarkers of response

The concentration of PAHs in sediments has been correlated with other organic chemicals such as PCBs and the metals Cd, Ni, Co and V. This substances has been also correlated with the induction of EROD activity in crabs the day 7 and 28 of exposure under laboratory conditions. Pb was linked with the induction of EROD activity after 28 days of field deployment, and Cu was also related to this biomarker the day 14 of laboratory exposure.

A relationship has been detected between the induction of EROD activity in crabs from laboratory bioassays the days 14 and 21. A similar correlation was observed in EROD activity measured in crabs from organisms collected from cages the days 7 and 14. The induction of the phase II detoxification enzymes in crabs after 28 days of exposure under laboratory and field conditions were linked in addition to the induction of this enzyme the day 21 of field deployment.



**Figure 4.** Histological sections of gills and hepatopancreas of the crab *Carcinus maenas* after 28-d exposure to the sediments: (A) Histological section of a control digestive gland (day 0); (B) Histological section of a control gill (day 0); (C) Histological section of hepatopancreas from a crab exposed to sediments from GR3' under field conditions; (D) Histological section of gill from a crab exposed to sediments from GR3' under field conditions; (E) Histological section of hepatopancreas from a crab exposed to sediments from GR3' under laboratory conditions; (F) Histological section of gill from a crab exposed to sediments from GR3' under laboratory conditions.

**Table1.** Spearman correlation (1:  $p<0.05$ ; 2:  $p<0.01$ ) results among chemical compounds bound to sediments and the induction of biomarkers the days 7, 14, 21 and 28

Chemicals in sediment										Biomarkers (laboratory assay)								Biomarkers (field assay)							
	PAHs	PCBs	Zn	Cd	Pb	Cu	Ni	Co	V	Gst 7	Gst 14	Gst 21	Gst 28	Erod 7	Erod 14	Erod 21	Erod 28	Gst 7	Gst 14	Gst 21	Gst 28	Erod 7	Erod 14	Erod 21	
Chemicals in sediment	PCBs	.90(2)																							
	Zn	0.41	0.22																						
	Cd	.86(2)	.68(1)	0.11																					
	Pb	0.12	0.04	0.19	-0.04																				
	Cu	-0.22	-0.36	-0.03	0.08	0.08																			
	Ni	.97(2)	.91(2)	0.29	.86(2)	0.20	-0.17																		
	Co	.93(2)	.84(2)	0.22	.87(2)	0.09	-0.34	.93(2)																	
	V	.87(2)	.78(1)	0.41	.68(1)	0.50	-0.01	.86(2)	.74(1)																
	Gst 7	0.45	.74(1)	-0.04	0.22	0.22	-0.23	0.52	0.46	0.53															
	Gst 14	-0.27	-0.03	-0.13	-0.33	0.16	-0.23	-0.13	-0.06	-0.29	0.43														
Biomarkers (laboratory)	Gst 21	-0.18	0.06	-0.26	-0.15	-0.12	-0.26	-0.12	-0.24	0.16	0.32														
	Gst 28	-0.04	-0.14	0.66	-0.31	-0.13	0.03	-0.24	-0.25	0.04	-0.21	-0.41	-0.43												
	Erod 7	.96(2)	.96(2)	0.26	.89(2)	0.14	-0.23	.99(2)	.87(2)	0.63	-0.18	-0.14	-0.20												
	Erod 14	-0.15	-0.29	-0.18	0.24	-0.13	.94(2)	-0.09	-0.27	-0.05	-0.40	-0.61	-0.13	-0.12	0.22										
Biomarkers (field)	Erod 21	-0.13	-0.11	-0.26	0.12	-0.22	0.66	0.02	-0.23	-0.17	-0.10	0.15	-0.33	0.10	.90(1)										
	Erod 28	.74(1)	0.63	0.13	.74(1)	-0.16	-0.52	.69(1)	.90(2)	0.422	0.21	-0.04	-0.11	-0.22	0.69	-0.43	-0.40								
	Gst 7	0.25	0.22	0.23	0.23	-0.03	0.11	0.20	-0.01	0.28	-0.12	-0.62	0.54	0.10	0.22	0.42	0.25	-0.14							
	Gst 14	-0.34	-0.30	-0.17	-0.36	0.24	-0.06	-0.16	-0.26	-0.33	-0.20	0.42	-0.08	-0.33	-0.23	-0.22	0.43	-0.28	-0.36						
Biomarkers (field)	Gst 21	-0.18	-0.17	0.34	-0.39	-0.58	-0.49	-0.36	-0.20	-0.41	-0.29	-0.13	-0.71	.73(1)	-0.41	-0.50	-0.42	0.09	-0.24	-0.18					
	Gst 28	-0.16	-0.20	0.31	-0.43	-0.29	-0.33	-0.34	-0.31	-0.21	-0.39	-0.48	-0.29	.80(2)	-0.34	-0.32	-0.30	-0.15	0.14	-0.08	.84(2)				
	Erod 7	-0.35	-0.05	-0.07	-0.52	-0.19	-0.39	-0.34	-0.41	-0.37	0.13	0.35	.90(2)	-0.02	-0.37	-0.31	-0.24	-0.34	0.46	-0.03	0.56	0.21			
	Erod 14	-0.55	-0.50	-0.02	-.7(1)	0.17	0.10	-0.59	-.7(1)	-0.29	-0.33	-0.34	-0.60	0.52	-0.57	0.25	0.04	-8(1)	0.07	0.27	0.32	0.71	.84(1)		
Biomarkers (field)	Erod 21	-0.30	-0.14	-0.38	-0.46	0.20	-0.11	-0.32	-0.29	-0.03	0.27	-0.03	-0.33	0.23	-0.25	-0.26	-0.31	-0.32	-0.49	0.00	0.12	0.34	-0.10	0.64	
	Erod 28	-0.19	-0.12	-0.12	-0.33	.73(1)	-0.08	-0.19	-0.20	0.21	0.26	0.11	0.23	-0.03	-0.20	-0.26	-0.50	-0.31	0.09	-0.15	-0.33	-0.10	0.25	0.36	

#### 4. Discussion

Crustaceans have the highest total P450 protein in the hepatopancreas, but also significant activity in green gland, gonads, and stomach (James, 1989), however The mechanisms by which xenobiotics activate gene expression leading to the increased production of new proteins such as P450s are not well understood in marine organisms (Snyder, 2000). Under laboratory conditions, the concentration of PAHs in sediment has been positively related to the induction of EROD activity the day 7 and 28 of exposure. This correlation also includes the metals Ni, Co, V and Cd which could be expected due to the presence of a complex mixture of contaminants, specially in sediments from the Bay of Algeciras. Under field conditions this correlation does not occur except for the induction of EROD activity the day 28 of deployment which is correlated with Pb. This metal has been often related to fuel used by old cars; in this case this EROD activity could be also related to other organic compounds not analyzed in the total PAHs included in this work.

Relationships between the same biomarker in consecutive weeks have been established what indicates that biomarkers follow a mechanism. In the case of GST activity correlation have been observed between laboratory and field assays showing that the stress that causes the activation of this enzyme is present in both kind of exposures.

In general, the fastest induction of biomarkers was observed in crabs exposed in laboratory to sediments from site GR3 with the highest content of PAHs in the sediment. In general, the other study sites present a later induction of biomarkers and normally EROD activity present peaks about 14 days after the beginning of the bioassays, and the same happens to GST activity; in the case of site E and F the GST activity keeps growing finding the maximum the last day of the bioassay.

Differences has been detected among the induction of biomarkers of exposure in crabs exposed to the areas of study. In general, in the case of the study sites located in the Galicia Coast, the induction of biomarkers is more important in caged organisms under field conditions. On the other hand organisms exposed to sediments from the Bay of Algeciras in laboratory are highest than those deployed in field. Results observed in histopathological analysis, use as biomarker of effect, indicate that organisms exposed in laboratory conditions are more damaged than those in field.

Previous studies showed how the disease status in the crab *Carcinus maenas* may be used as a high-level indicator of ecosystem health.(Stentiford and Feist, 2005). Histopathological observation showed that crabs exposed to sediments from the Bay of Algeciras under laboratory conditions were the most damaged followed by and crabs exposed to sediments from the Bay of Corme-Laxe, and finally organisms from the Cies treatment which showed mainly alterations due to general environmental stress. The relationship between pollutants and pathologies in target tissues has been previously reported (Ortiz-Delgado et al., 2007). In this case, under laboratory conditions, the highest amount of PAHs in the sediment is, the most histological lesions in crabs are observed, as in the case of site GR3 from the Bay of Algeciras. The alterations shown in crabs exposed to sediment from GR3 has been previously observed in different marine invertebrate or vertebrate species exposed to different inorganic or organic contaminants, parasitic or infectious diseases, nutritional stress, or physico-chemical disorders (Rodriguez de la Rua et al., 2005; Ortiz-Delgado et al., 2007).

Although a causal relationship must exist between exposure to contaminants and biological effects, such a causal link does not necessarily hold between the two types of biomarkers (biomarkers of response and biomarkers of effect), except if biomarkers share a common metabolic pathway. (Lafontaine

et al., 2000). In this case histopathological lesions are higher in organisms exposed to the sediment with the highest content of PAHs; in addition, the detoxification system induced by the EROD activity the day 7 and 28 also correlates with this contaminant. Previous studies with crabs also found relationships between PAHs and the induction of EROD activity (Fossi et al., 2000; Martín-Díaz et al., 2004)

## **5. Conclusions**

The following conclusions can be drawn from the results of the present study:

1. The concentration of PAHs in sediments has been correlated to the induction of EROD activity in the hepatopancreas of the exposed crabs under laboratory conditions.

2. In the case of organisms deployed in cages in the Bay of Algeciras, effects of contaminants bound to sediments decrease considerably as indicated by biomarkers of exposure and effect. This could be related to a diminution of bioavailability of contaminants due to the water removal mainly produced by the high influence of tides.

3. Organisms deployed in the Galician Coast probably present other sources of stress not related to sediment as it has been shown in the low biomarker activities under laboratory assays and higher activities under field conditions, specially in crabs deployed in the Bay of Corno-Laxe. However the sources of stress did not produce significant histopathological effects.

4. The importance of carrying out kinetic approaches of biomarkers of response in marine invertebrates has been shown; the incorporation of biomarkers of effect and chemical data in addition to the combination of both

field and laboratory assays to the kinetic study helps to elucidate possible sources of contamination.

## **6. Acknowledgements**

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# **A comparative analysis of macrobenthic community structure in relation to different oil contaminated sediments: the Galician Coast (acute, *Prestige* oil spill) and the Bay of Algeciras (chronic oil spills).**

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## **Abstract**

The macrobenthic fauna and chemicals concentrations in sediments were examined in the Galician Coast (NW Spain) along three years after the *Prestige* oil spill (November, 2002). Results obtained points to an initial impact to the benthic community due to the fuel oil although a recovery of the environmental quality was observed four years after the accident. Selected sites located in the Galician Coast were compared to the status of sediments chronically affected by oil spills in the Bay of Algeciras and those not affected in the Bay of Cadiz in order to assess the recuperation capacity of the ecosystem after an acute impact. The methodology employed includes univariate analysis using conventional community descriptive parameters and the numerical contribution of major taxonomic groups. Results obtained in the areas of study have been linked with the physicochemical characterization of sediments with the purpose of identifying the cause and source of contaminants.

*Keywords: sediment alteration, macrobenthic populations, community, environmental degradation, PAHs.*

## **1. Introduction**

The oil tanker Prestige broke down in the Galician Coast (NW Spain) on November 2002 and approximately 60 000 tonnes of heavy fuel oil were released into the surrounding waters, resulting in the contamination of more than 1000 km of coastline; this accident supposed one of the major ecological catastrophes of the Iberian Peninsula. Results of work investigating the impact of oil spills on a variety of biological components have confirmed the effects in a wide range of habitats and species (Peterson et al., 2001).

The assessment of *in situ* alteration of residential community structure has been often performed to determine the effects of pollutants in the coastal environment (DeIvals et al., 1998a., Gómez-Gesteira and Dauvin, 2005). Field data on the communities living in the sediments allow establishing whether there is observable pollution-induced degradation effect in the biota (Chapman et al., 1991, Chapman, 2007). Three symptoms of stress reduced diversity, retrogression to opportunistic species, and reduced size of individuals are documented for a wide variety of natural and anthropogenic stresses in marine environments (Gray, 1989). Identification of these symptoms in a benthic assemblage may signal a change in environmental conditions resulting from anthropogenic influences (Newell et al., 1999). Previous studies (cited in Blanchard et al., 2002) indicated that some benthic organisms can respond to non-toxic fractions of crude oil as they would to other forms of organic enrichment (e.g., Weston, 1990) and this is suggested by the response of an increase in some polychaete species.

Multivariate analysis appears to be an especially sensitive tool for detecting change in the structure of the faunal community (Warwick and

Clarke., 1991). The integration of field data with chemicals analysis permits establishing the possible causes and sources of the benthic alteration.

The scope of this study is to examine the recovery of benthic community after the *Prestige* oil spill and to compare the environmental status of the Galician Coast after 4 years of the spill with the macrobenthic structure analyzed in Bay of Algeciras that is chronically impacted by different oil spills and with that analyzed in the Bay of Cadiz considered not contaminated neither polluted by this kind of activities or contaminants.. The comparative analysis will be carried out by using univariate and multivariate methods.

## **2. Material and methods**

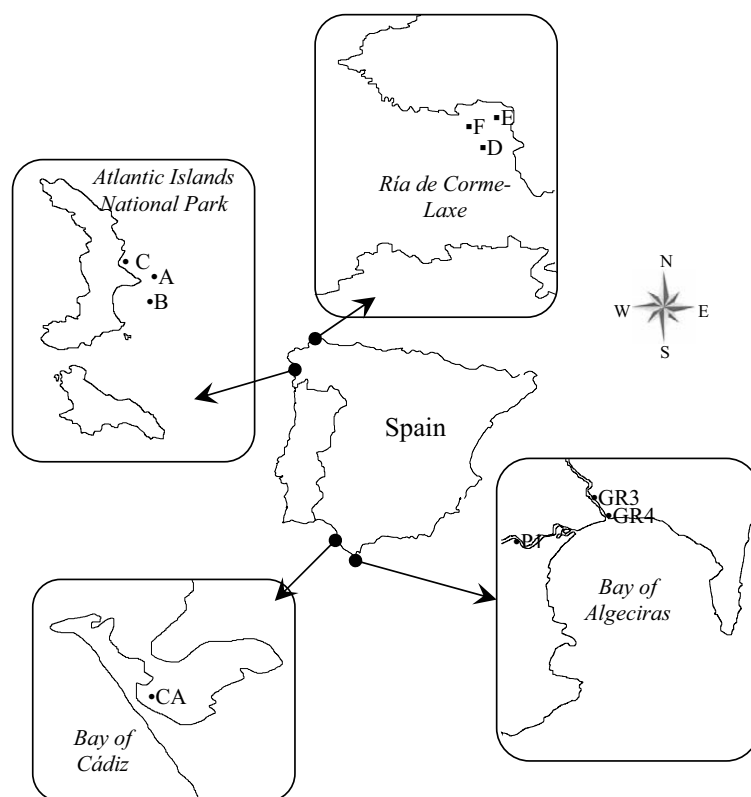
### **2.1. Sites description**

Figure 1 shows the selected sites in the Galician Coast (NW Spain) were located in the Cies Island (A, B and C) in the Atlantic Island National Park (AINP) and in the Bay of Corme-Laxe (D, E and F). Both areas were importantly affected by the *Prestige* oil spill and are considered of high ecological importance. The second area of study was the mouth of the River Palmones (P1) and Guadarranque (GR3 and GR4) in the Bay of Algeciras (S Spain); this place was selected because is highly industrialized and there are a large number of petrochemical activities which comprise several accidental oil spills. A reference site, CA, widely characterized by different ecotoxicological studies (Del Valls et al., 1998b, Riba et al., 2004, Martín-Díaz et al., 2005; Cesar et al. 2007; Morales-Caselles et al., 2007) was selected in a clean area in the Bay of Cádiz (S Spain).

### **2.2. Sample collection**

Sediment samples were collected with a 0.025 m<sup>2</sup> van Veen grab. Only grabs that achieved adequate penetration (2/3 of total volume) to collect the superficial 5 cm of the sediment and that showed no evidence of leakage or

surface disturbance were retained for the study. For the benthic infaunal samples, the entire contents of the grab including overlying water, were wet sieved at the study site with a 0.5 mm stainless steel mesh. Residues were gently washed, placed in polyethylene bottles, preserved with 10 % buffered formalin and stained with Rose Bengal. Sediments for chemical analyses were collected and transported to a cooler. Sediment samples were kept in dark at 4 °C prior to analysis.



**Figure 1.** Map of the coastal area of Galicia showing the locations of the sampling stations. A, B and C refers to the stations located in the Cies Island in the Atlantic Island National Park and D, E and F to those in the Bay of Corme-Laxe. The stations located in the Bay of Algeciras are GR3, GR4 and P1. The station CA located in the Bay of Cadiz corresponds to the sediment used as reference.

### 2.3. Laboratory analysis

The organisms collected in the study sites were separated from the remaining sediment, sorted and identified to the lowest possible taxon level (species level, or family in case of Polychaeta). Identifications to the family level were considered adequate measures of faunal composition for the purposes of this study.

Polycyclic aromatic hydrocarbons (PAHs) bound to sediments were analyzed by using a gas chromatograph equipped with mass spectrometer (GC/MS) (USEPA, 1994). Briefly dried samples were Soxhlet extracted with n-hexane for 18 h, and the extracts were isolated by column chromatography on Florisil®. PAHs were eluted and their fractions were dried in a rotating evaporator and re-dissolved in isooctane. Aromatic fractions were analyzed on a HewlettePackard (HP) 5890 Series II gas chromatograph coupled with an HP 5970 mass spectrometer. PAHs were analyzed by GC-MS using selected ion monitoring (SIM). The analytical procedure showed agreement with the certified values of more than 90%.

Trace metal were analyzed as described by Casado-Martínez et al. (2006); briefly, 2.5 g of sediments (<0.065 mm) were placed in Teflon containers and were digested in microwave (400W, 15 min, twice) with HNO<sub>3</sub> 2N. The extracts were purified by passing through a C-18 column and metals analyses were performed by anodic voltamperimetry (-Zn, Cd, Pb, Ni, Co and Cu- Metrohm Application Bulletin N° 147; - V- Metrohm Application Note N° V-81). For Hg the cold vapour technique was used and was quantified using atomic absorption spectrometry. The analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) and showed a recovery greater than 90% of the certified concentration.

Organic carbon content was determined using the method of Gaudette et al. (1974) with the El Rayis (1985) modification. For sediment grain size, an aliquot of wet sediment was analyzed using a Frisch laser particle sizer (model Analysette 22) following the method reported by DelValls and Chapman (1998b).

#### 2.4. Data analysis

Descriptive statistics were used in order to describe the the macrobenthic community at each site; univariate methods included classical community descriptive parameters, as species richness (Margaleff's  $R$ ), Shannon-Wiener diversity ( $H'$ ), evenness (Pielou's  $J$ ), and Simpson's dominance ( $D = 1/\lambda'$ ) (Gómez-Gesteira and Dauvin, 2005; Choueri et al., submitted). Since numerical contribution of major taxa is widely utilised to evaluate pollution effects (DelValls, 1998a; Chapman et al., 1996), an abundance analysis was carried out by calculating the proportion of major taxa's (Polychaeta, Mollusca and Crustacea) abundance to the total abundance for each sample.

Multivariate analysis was carried out with in an attempt to link contamination with benthic alteration parameters; the principal component analysis (PCA) was used as the extraction procedure which is a multivariate statistical technique to explore variable distributions (Riba et al., 2003). The objective of PCA is to derive a reduced number of new variables as linear combinations of the original variables. This provides a description of the structure of the data with the minimum loss of information. A hierarchical classification technique by means of a Cluster analysis was performed to determine the percentage disagreement among the study sites taking into account the physicochemical and biological variables, results were displayed in a dendrogram.

### 3. Results and discussion

#### 3.1. Evolution of the benthic community and chemical concentration in sediments from the Galician coast (2004-2006)

Three surveys were carried out in the AINP after the *Prestige* oil spill (2004, 2005 and 2006) whereas two (2005 and 2006) were performed in the Bay of Corme Laxe. The decrease in the abundance of the macroinfauna observed after the spill appear to reflect the losses due to oiling toxicity or indirect effects of oiling and clean-up (Junoy et al., 2004). Table 1 shows the decrease in the concentration of PAHs in sediments 4 years after the *Prestige* oil spill in all study sites (Morales-Caselles et al., accepted). For all stations the number of species increased from the first survey till the last one; this increase was especially important in sites B and C from the AINP. The specific richness also increased in sites selected in the Cíes Islands whereas it kept similar in D and F and decreased in site E from Corme-Laxe. Diversity presented a diminution in site E while an increase of this parameter was observed in the other study sites. In general, all stations presented a high population of polychaete after the spill which decreased along the time whereas other taxons such as molluscs and crustacean increased. It is known that the abundance of opportunistic taxa such as polychaete increase in the presence of petroleum hydrocarbons (Feder and Blanchard, 1998) whereas declines in benthic amphipods also occurred following the *Amoco Cadiz* (Dauvin, 1982) and the *Aegean Sea* oil spill (Parra and López-Jamar, 1997). Univariate analyses of benthic data from our study showed the trends (i.e. low faunal abundance, relatively high dominance and low diversity) observed following oil spills (Feder et al., 1998), what indicates that the environment was negatively impacted by the accident of the tanker *Prestige*; however, the variation of the benthic parameters along the time and the diminution of the concentration of PAHs bound to sediments points to a

recovery of the environmental quality in the following years after the oil spill, what has been confirmed by other authors (Serrano et al., 2006).

**Table 1.** Summarized results of the concentration of PAHs in sediments the and benthic alteration parameters measured for the study of the environmental quality in the Cies Island in the Atlantic Island national Park 2004-2006 (first survey: A-1, B-1, C-1; second survey: A-2, B-2, C-2; third survey: A-3, B-3, C-3) and the Bay of Corme-Laxe 2005-2006 (second survey: D-2, E-2, F-2; third survey: D-3, E-3, F-3).

Stations	Parameters measured						
	PAH $\mu\text{gKg}^{-1}$	Species Nº	Specific richness	Diversity	Molusca %	Polychaeta %	Crustacea %
A-1	390	3.0	1.8	1.5	0.1	53.0	33.3
A-2	119	5	12.0	4.3	2.4	21.0	34.5
A-3	108	7.09	28.5	5.1	15.3	20.0	37.0
B-1	2120	2	1.2	1.0	0.1	100.0	0.1
B-2	366	12	5.9	5.2	9.9	56.2	15.4
B-3	67	47	33.9	5.0	28.4	21.5	41.0
C-1	420	9.0	15.3	2.9	22.2	33.3	33.3
C-2	239	30.0	50.9	4.5	26.7	26.7	43.3
C-3	n.d.	25.0	42.4	4.3	39.1	21.7	39.1
D-2	537	9	25.7	2.9	33.3	33.3	33.3
D-3	38	10	28.6	3.0	30.0	20.0	50.0
E-2	558	12	66.7	5.0	2.0	30.6	100.0
E-3	52	12	32.1	3.0	40.1	22.2	51.4
F-2	820	15	55.6	2.3	40.0	26.7	33.3
F-3	323	13	48.2	2.9	15.4	23.1	61.5

In order to elucidate if there is still degradation of the environment in the Galician Coast four years after the spill, the evaluation of the sediment quality, including physicochemical and benthic parameters was compared with the situation of the area of the bay of Algeciras, chronically affected by oil spills.

### 3.2. Physicochemical characterization of sediments from the Galician Coast and the Bay of Algeciras (2006)

Summarised results for the chemical data and physical characterization of the sediments are shown in Table 2. Stations from the Bay of Algeciras presented the highest concentrations of organic carbon and fines in the sediments. The highest concentrations of PAHs were observed in the stations located in the Bay of Algeciras whereas GR3 presented the highest values of Pb and Ni often related to hydrocarbons. The fact that there is a petrogenic industry closed to this point and the presence of bunkering activities in the Bay could explain the input of hydrocarbon in the area. On the other hand neither the reference station nor the site C showed presence of PAHs in their sediments. No general pattern was observed for other contaminants in the studied areas from Corme-Laxe and Cies Island. In general, sites from the Bay of Algeciras present the highest content of fines and organic carbon in their sediments.

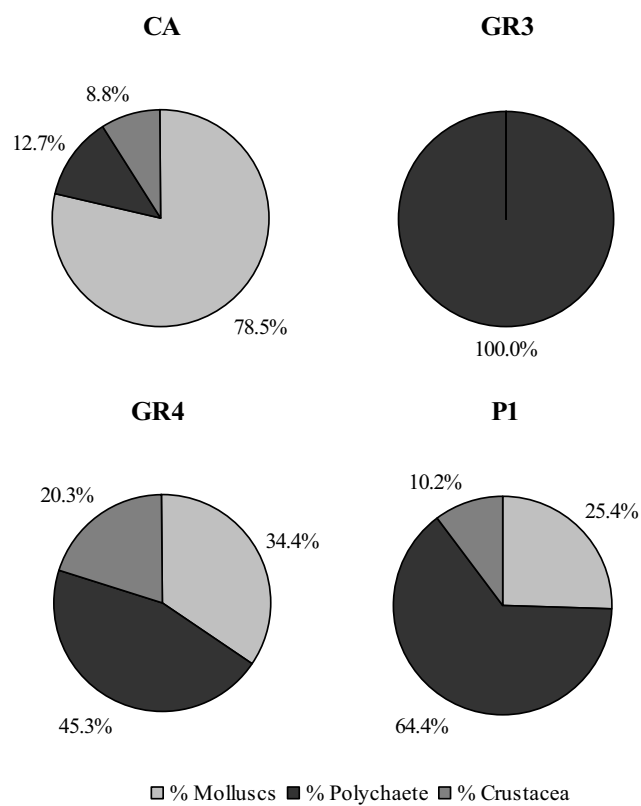
### 3.3. The benthic community in the Galician Coast and the Bay of Algeciras (2006)

The description of the benthic community differs depending on the sampled area. For sediments collected in the reference station (CA) Molluscs were the best represented taxon (78.5%) followed by Polychaete (12.7 %) and Crustacea (8.8 %) (Figure 2). Surveys in the Bay of Algeciras showed a prevalence of polychaete, 45.3 % in GR4, 64.4 % in P1 whereas in GR3 all the community was made up by polychaete. The dominance distribution of taxa in Bay of Algeciras reveals that pollutant resistant groups, according Grall and

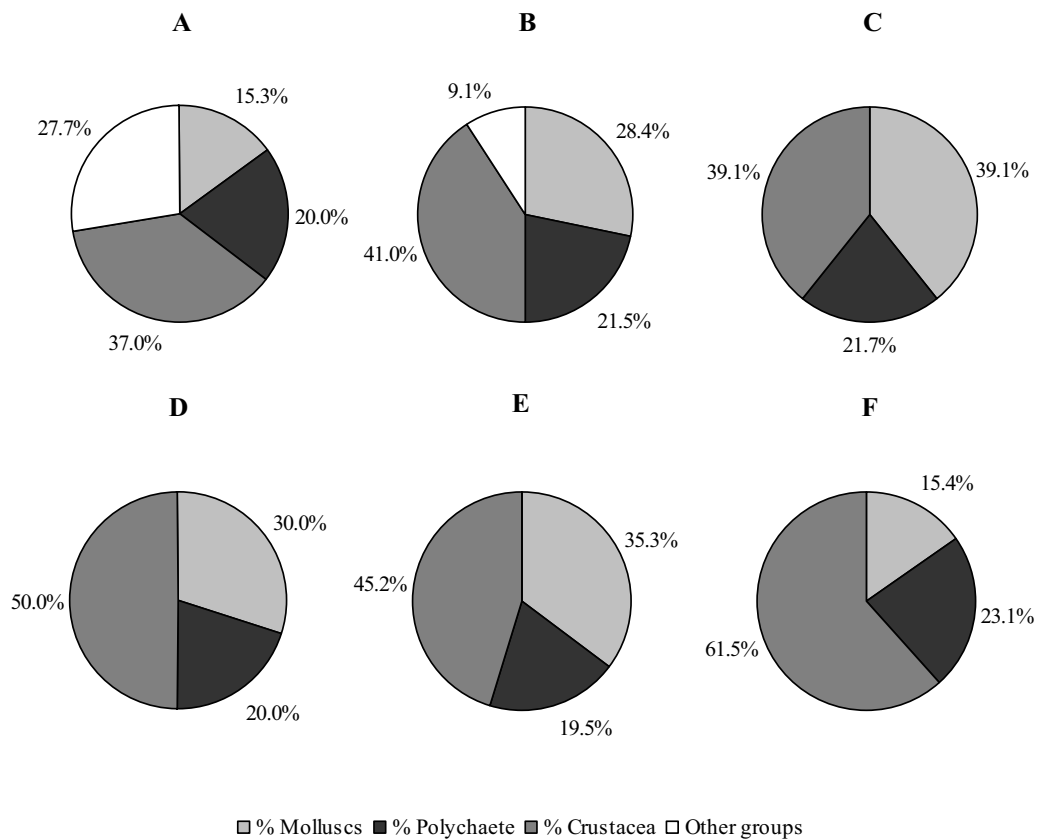
**Table 2.** Total PAHs, PCBs and metal concentration (Zn, Pb, Cu, Ni, and Hg) and benthic parameters (number of species, specific richness, diversity and dominance) measured in the sediments from Galicia: Atlantic Islands National Park (A, B, C), Corme-Laxe (D, E, F); the Bay of Algeciras (GR3, GR4 and P1) and the Bay of Cadiz (CA) used as the reference station.

study sites	Physicochemical analysis								Benthic alterations			
	O.C. %	Fines %	Zn mgKg <sup>-1</sup>	Pb mgKg <sup>-1</sup>	Cu mgKg <sup>-1</sup>	Ni mgKg <sup>-1</sup>	Hg mgKg <sup>-1</sup>	PAH µgKg <sup>-1</sup>	Species N.	Specific richness	Diversity	Dominance
CA	1.07	2.50	21.3	2.28	6.98	0.06	n.d.	n.d.	14	2.6	1.64	0.66
A	0.28	4.32	377	1.50	5.20	13.3	0.70	108	7.09	28.5	5.1	0.50
B	0.26	2.81	91.0	0.90	1.40	2.40	0.80	67.0	47	33.9	5	0.10
C	0.30	2.76	164	0.85	1.40	4.50	0.60	n.d.	25	42.4	4.3	0.06
D	0.31	3.79	25.0	3.70	0.70	1.70	2.00	38.0	10	28.6	3	0.15
E	0.37	5.50	19.9	7.30	0.43	1.50	2.10	52.0	12	32.1	3	0.19
F	0.65	5.95	271	5.90	4.20	5.70	3.40	323	13	48.2	2.9	0.20
GR3	2.15	69.4	138	21.6	5.01	74.7	1.04	2961	0.67	0.0	0.0	0.0
GR4	3.19	59.3	35.3	6.21	3.67	13.1	0.25	802	4.67	1.2	1.29	0.72
P1	3.86	35.4	56.7	12.3	75.2	13.3	0.65	641	4.67	1.3	1.24	0.68

Glémarec (1997) classification, are more abundant. Polychaeta (Capitellidae and Nereidae) was the most common taxa, followed by Mollusca (only pollution-resistant species, as Cerastoderma edule and Abra tenuis) (Choueri et al., submitted). This pattern of abundance has been shown in areas affected by oil spills (Parra and López-Jamar, 1997; Serrano et al., 2006). Other authors consider that these taxa appear to be responding to moderate enhancement of the benthos by residual hydrocarbons in effluents as a food source (Blanchard et al., 2002). Crustacea was the most frequent taxa in all the sites located in the Galician Coast, 37.0 % in A, 41.0 in B, 39.1 % in C, 50.0 % in D, 51.4 % in E and 61.5 % in F. The highest number of species was detected in the sites B (47) and C (25) from the Cies Islands (14), followed by the reference station (CA) whereas the lowest number was observed in the Bay of Algeciras. Benthic species of slow growth and with slow recovery capability, mainly crustaceans and echinoderms, show a high sensitivity to oil exposure (Serrano et al., 2006). With regard to other population parameters, species richness ranged from 48.2 in F to 0 in GR3, while the highest diversity was shown in the AINP. No significant changes in benthic community structure, characterized by species richness, individual abundance, and diversity were determined after the Braer oil spill (Kingston et al., 1995) what agrees with the affirmation that few valid generalizations about ecological effects can be applied to most spills (Junoy et al., 2004). No diversity, therefore no dominance was found in the station GR3 in Algeciras. Changes in mean abundance, biomass, or diversity at a station that were unlike, or out of phase with, the trends observed for other stations (an interaction effect), indicate possible influences by sources other than natural factors (Jewett et al., 1999; Blanchard et al., 2002). However, low values of species richness and low diversity and high dominance of few better adapted species are expected for aquatic ecosystems like estuaries or a mouth of a river, where the variation of environmental conditions (salinity, pH, temperature) is stressing to the biota (Choueri et al., submitted).



**Figure 2.** Distribution of the main taxa in sediments from stations selected in the Gulf of Cádiz.



**Figure 3.** Distribution of the main taxa in sediments from stations selected in the Galician Coast.

### 3.4. Linking physicochemical characterization with benthic alteration

A principal components analysis was performed towards two objectives: to elucidate if the benthic alteration was due to pollutants bound to sediments and to determine which contaminants are the cause of the environmental impact.

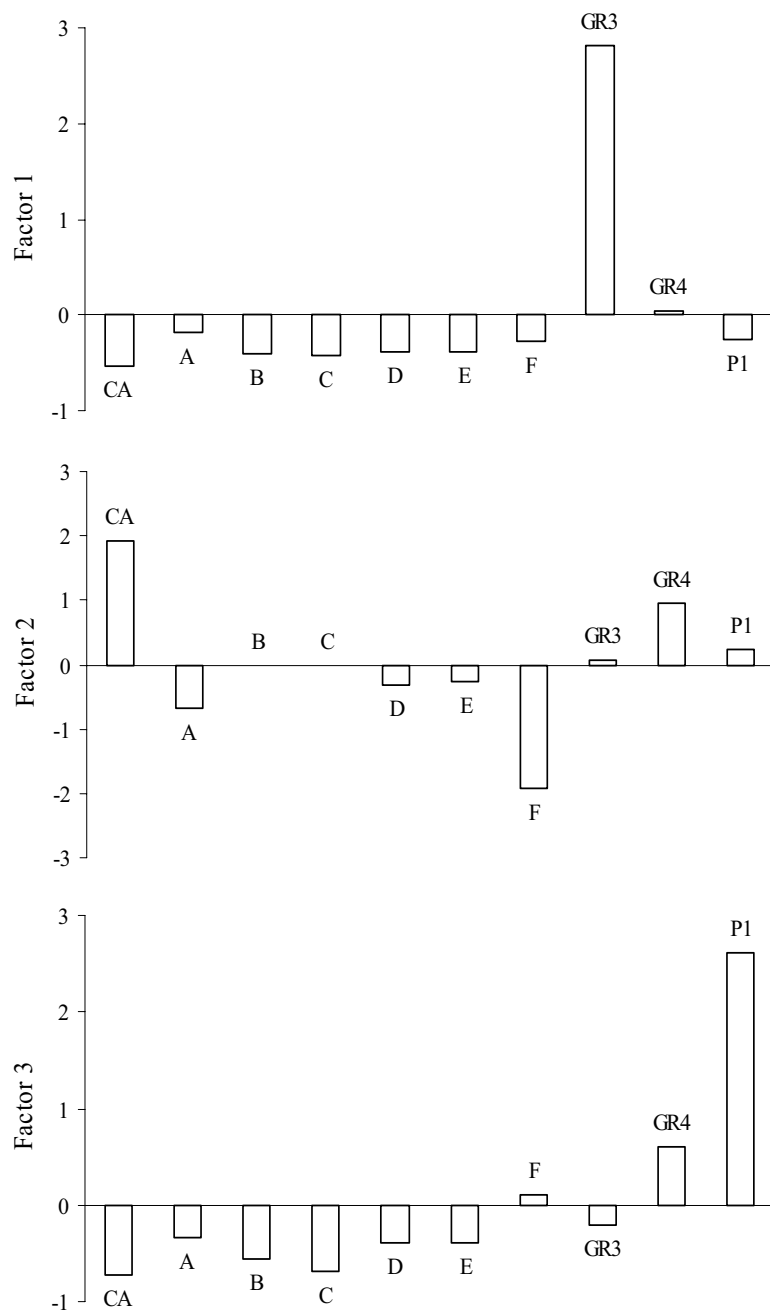
The multivariate analysis shows that the original variables can be grouped in three new factors that explain an 89 % of the total variance (Table 3). The first factor (58.5 %) links the presence of Pb, Ni and PAHs bound to sediment, concentration of organic carbon and fines, with all the parameters related with the benthic alteration. These contaminants are usually components of fuel oils what suggest that a source or sources of these compounds are producing an environmental impact in some of the studied areas. This factor has mainly prevalence in the station GR3 from the Bay of Algeciras (Figure 4) meaning that there is environmental degradation in this site due to the input of contaminants related with oil spills. The station GR4 presents the influence of this factor (in minor degree than GR3), what also means that the alteration of the benthic community is due to the spills of oil that often occur in this area.

Both locations are close to a petrochemical industry what suggest that there is a chronic input from this source in addition to accidental spills and other activities in the Bay of Algeciras. The second factor (21.0 %) correlates, with negative loading, the metals Zn and Hg with the population of molluscs, whereas opposite relationship are shown among these contaminants, the percentage of organic carbon in the sediment and some benthic parameters such as the specific richness and the population of crustacean. This factor, could be explained as the potential stress that these contaminants might produce to the environment in those sites with negative loading (Figure 4) with mainly prevalence of station F and followed by D, E (Corme-Laxe) and A (Cíes Island). The positive loading presented in the reference site and related to the alteration

of the population of crustacean it is probably related to the high activity of fishermen in the area who collect different species of this taxon. The third factor (9.4 %) links the concentration of Pb and Cu bound to sediment with the percentage of fines and organic carbon in the sediment and with the benthic alterations determined by the specific richness and the disturbance of the populations of polychaeta and crustacean. This relationship indicates that these metals are producing environmental degradation and can be considered a risk for the benthic community. Factor 3 presents positive loading in sites P1 and GR4 from the Bay of Algeciras and station F in Corme-Laxe.

**Table 3.** Sorted rotated factor loadings of 15 variables for the three principal factors resulting from the multivariate analysis of results obtained from the chemical analysis and the benthic alteration parameters.

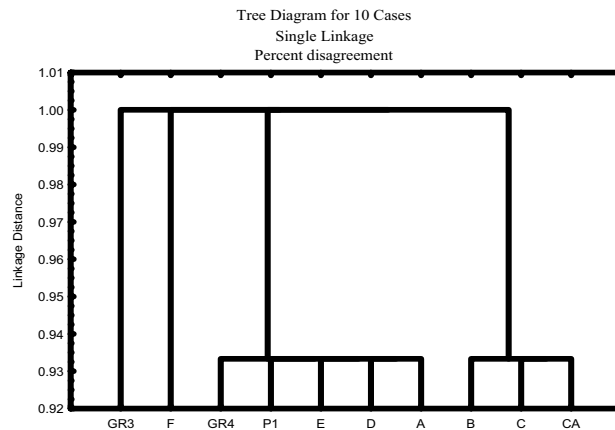
	<b>Factor 1</b>	<b>Factor 2</b>	<b>Factor 3</b>
	<i>58.5</i>	<i>21.0</i>	<i>9.4</i>
<b>Zn</b>	—	-0.63	—
<b>Pb</b>	0.86	—	0.42
<b>Cu</b>	—	—	0.92
<b>Ni</b>	0.99	—	—
<b>Hg</b>	—	-0.83	—
<b>PAH</b>	0.98	—	—
<b>O.C.</b>	0.32	0.37	0.84
<b>Fines</b>	0.77	—	0.44
<b>Species N.</b>	0.99	—	—
<b>Specific richness</b>	0.44	0.73	0.44
<b>Diversity</b>	0.99	—	—
<b>Dominance</b>	0.99	—	—
<b>Molluscs</b>	0.58	-0.69	—
<b>Polychaete</b>	0.87	—	0.47
<b>Crustacea</b>	0.55	0.72	0.34



**Figure 4.** Factor loadings for the three principal factors resulting from the multivariate analysis of results obtained from the chemical analysis and the benthic alteration parameters.

Results obtained with the MAA indicate that fuel oil is no more affecting the benthic community of the AINP and the Bay of Corme-Laxe in the Galician Coast, whereas sites evaluated in the Bay of Algeciras have shown a deep impact of chronic oil spills which are the cause of the important environmental degradation of the area. In addition, although no pollution was observed in the reference site (CA), the high influence of fishermen could be considered a threat for some taxons such as crustaceans. The presence of metals bound to sediments mainly in Algeciras and Corme-Laxe and some sites of the AINP can be considered a risk for the benthic community and alteration of the environment can be expected.

Figure 5 present the results of the cluster analysis and shows the heterogeneity of the areas of study. Sites from the Bays of Algeciras and Corme-Laxe appear further from the reference site whereas locations from the AINP are grouped close to the reference station. In this sense, the behaviour of the Cies Islands is quite similar to a reference site whereas sites from Corme-Laxe, especially station F presents an environmental alteration lower but nearer to the degradation of the Bay of Algeciras.



**Figure 5.** Classification tree of the study sites based on the cluster analysis. The station CA corresponds to the reference site. A, B and C refers to

the stations located in the Cies Island in the Atlantic Island National Park and D, E and F to those in the Bay of Corme-Laxe. The stations located in the Bay of Algeciras are GR3, GR4 and P1.

#### **4. Conclusions**

This report shows the recovery of the benthic community from the Galician Coast four years after the spill of the tanker Prestige (2002). The data obtained were compared with those from an area chronically affected by oil spills, the Bay of Algeciras and an area not contaminated in the Bay of Cadiz. A multivariate analysis was performed to determine the cause of the benthic alterations. Results obtained show a high environmental degradation in sediments from the Bay of Algeciras which suffers the input of different contaminants but mainly fuel oil. Biological stress was also observed due to sources of metal contamination in all the studied areas but mainly in Algeciras and the Bay of Corme-Laxe.

The present study shows the importance of the combination of physicochemical and biological data to estimate the health status of the sediments. Besides, the results achieved suggest that benthic community is able to recuperate a few years after a major oil spill whereas sediments affected by low albeit continuous inputs present a chronic environmental degradation.

#### **5. Acknowledgments**

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## Capítulo 5.

### Aplicación de un método integrado para la caracterización de sedimentos afectados por vertidos de petróleo

En la actualidad sabemos que para estudiar la calidad ambiental de los sedimentos no basta con determinar los niveles de contaminantes presentes en la matriz, existen aspectos, tales como la biodisponibilidad de los contaminantes una vez incorporados al sedimento, la acción concomitante de las condiciones fisicoquímicas del medio y la posibilidad de efectos sinérgicos o antagónicos con otros contaminantes, que hacen que estas medidas disten mucho de ser una valoración objetiva de la "salud ambiental" de esos sistemas (DelValls, 2006). Así pues el riesgo potencial de una sustancia química dependerá de (Chapman, *Master lessons*):

**Bioaccessibilidad:** la fracción o matriz que potencialmente puede resultar disponible para el organismo.

**Biodisponibilidad:** la sustancia que inmediatamente es disponible para ser incorporada por los organismos.

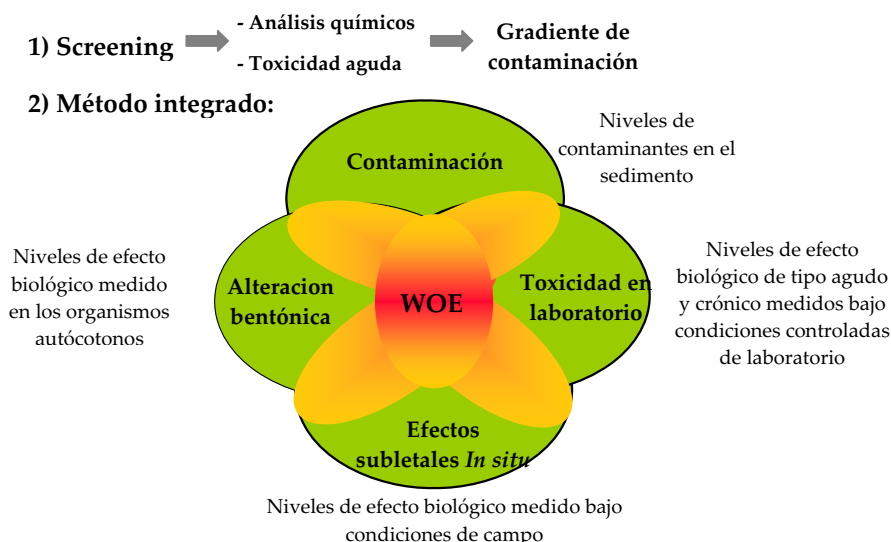
**Bioabsorción:** Lo que realmente es incorporado por el organismo.

**Biorreactividad:** Lo que realmente es capaz de causar toxicidad (la fracción bioabsorbida menos la fracción depurada, secuestrada internamente o utilizada por el organismo para cubrir sus necesidades)

Se han aplicado distintas metodologías para evaluar la calidad ambiental de sedimentos sin limitarse a los análisis químicos. Estas aproximaciones incluyen medidas de la bioacumulación en organismos seleccionados como indicadores de contaminación, los efectos letales y/o subletales observados tras la exposición de organismos a sedimentos ‘supuestamente’ contaminados, las modificaciones de la estructura de las comunidades que produce la contaminación, etc. (DelValls, 2006). Sin embargo, cada una de estas propuestas presenta sus limitaciones.

Con el fin de superar las limitaciones que presentan las metodologías se propuso la realización de un estudio integrado en el que se incluyan distintas tecnologías de forma que haya una aproximación lo más certera a la realidad. Para ello se emplean las llamadas líneas de evidencia (lines of evidence, LOEs) que incluyen cada una de las metodologías aplicadas de forma individual para posteriormente realizar una integración global de los resultados dentro del llamado “Weight of Evidence (WOE) approach” o “peso de la evidencia”, contrarrestando así la subjetividad y la arbitrariedad. Las conclusiones proporcionadas por cada medida individual, dentro del método integrado, son consideradas en relación con las que ofrecen los otros componentes del método. De esta manera, se evalúa la correlación entre los resultados obtenidos por técnicas distintas y se dispone de unos resultados más cercanos a la realidad que cuando se aplican esas mismas técnicas de forma individualizada (DelValls, 2006). Este tipo de metodología se ha aplicado satisfactoriamente tras episodios de vertidos contaminantes (Ej. DelValls and Chapman, 1998; Chapman, 2000; Borgmann et al., 2001; Riba et al., 2004; Lee et al., 2006).

Una de las principales ventajas de la utilización de un método integrado se encuentra en la posibilidad de caracterizar las llamadas zonas grises ("gray zone"). En torno al 70% de las áreas litorales y de estuario pueden incluirse dentro de estos ecosistemas, que poseen un grado de polución intermedio entre las zonas claramente alteradas y las que pueden ser consideradas como no estresadas (DeIvals, 2006).



**Figura 5.1.** Representación esquemática del desarrollo del modelo integrado aplicado para el seguimiento del impacto del vertido del petrolero 'Prestige' y su comparación con la calidad ambiental en zonas afectadas por vertidos de petróleo de tipo crónico (continuos) y con ausencia de influencia por este tipo de vertidos (Bahía de Cádiz). En una primera fase de aplicación del modelo se desarrolla la fase inicial de 'screening' con la aplicación de sólo dos LOEs sobre un número extenso de estaciones. El resultado de la aplicación de esta primera fase va a permitir la selección de un número menor de estaciones sobre las que se va a desarrollar el modelo integrado de forma completa. Ésta incluye cuatro líneas de evidencia: contaminación, toxicidad, alteración 'in situ' y bioacumulación y biomagnificación.

En este capítulo se presentan tres trabajos realizados consecutivamente. En el trabajo XIV, se lleva a cabo un estudio integrado de la calidad de los sedimentos del Parque Nacional de las Islas Atlánticas y la Bahía de Corme-Laxe a lo largo de tres años. Para realizar este estudio se empleó una metodología clásica basada en 3 líneas de evidencia: a) Análisis químicos de los sedimentos, b) Toxicidad aguda bajo condiciones de laboratorio, y c) alteración *in situ* de la comunidad bentónica. Este estudio pone de manifiesto la recuperación de la “salud ambiental” cuatro años después del vertido del *Prestige*, principalmente en las islas Cíes, aunque detecta cierta contaminación metálica potencialmente peligrosa sobretudo en la Bahía de Corme-Laxe. Además, la aplicación de un análisis de la varianza a los resultados obtenidos en la integración de los datos permitió establecer diferencias significativas entre las estaciones de estudio.

Una vez realizado el estudio presentado en el trabajo XIV se decidió ampliar el número de líneas de evidencia, manteniendo las anteriores e incorporando los resultados obtenidos en los experimentos subletales, tanto en campo como en laboratorio. De esta forma el trabajo XV muestra esta integración donde se hace hincapié en la importancia del uso de biomarcadores para evaluar calidad ambiental de los sedimentos y los riesgos potenciales.

El último trabajo del capítulo, el XVI, emplea la información obtenida a lo largo de la tesis doctoral y presentada en los anteriores capítulos para dar respuesta al planteamiento inicial del estudio, comparar la calidad ambiental de los sedimentos de dos áreas afectadas por vertidos de petróleo. Para ello se lleva a cabo una integración completa con diversas líneas de evidencia para clarificar el estado de los sedimentos de la costa gallega y de la Bahía de Algeciras.

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# Using a classical Weight-of-Evidence approach for 4-years' monitoring of the impact of an accidental oil spill on Sediment Quality

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## Abstract

In the present report, the successful application of a Weight of evidence approach (WOE) to sediment quality assessment during a four year impact period following an oil spill is discussed. The study assesses the sediment quality on the Galician Coast (NW Spain) which was impacted by an accidental spill associated with the sinking of the tanker Prestige (2002). The assessment is based on three lines of evidence: physicochemical characterization of the sediments; determination of acute toxicity by conducting sediment toxicity tests and benthic alteration including taxonomic identifications along with community descriptive statistics. The data obtained were integrated using a WOE approach by means of two different methodologies: multivariate analysis and ANOVA-based pie charts. Results confirm that PAHs related to the Prestige oil spill are the main contaminant associated with biological effects in the area which has since recovered from the initial acute impact. Also, the WOE allowed the identification of metal contamination not previously described in the area responsible for toxicity in sediments analyzed. In addition, the

methodology proposed to link the 3 lines of evidence results shows the use for the first time of an objective indice based on factor analysis which allows pollution of the sediments studied to be qualitatively and quantitatively evaluated while demonstrating the WOE approach to be recommendable in monitoring environmental quality.

*Keywords: PAHs, contamination, toxicity, WOE, Sediment Quality Triad*

## **1. Introduction**

Chemical measurements in the environment provide information on contamination (substances present where they would not normally occur, or above natural background concentrations), but they do not provide information on pollution (contamination that causes adverse biological effects in the environment) (Chapman, 2007). Chemical analyses are an important tool in sediment quality assessment, however the information obtained does not report on the consequences that chemicals have on the organisms exposed to them. Biological effects established based on laboratory tests to determine toxic responses in combination with field data on the communities living in the sediments allow it to be established whether there is observable pollution-induced degradation effect a given set of biota (Chapman et al., 1991).

Weight of evidence (WOE) investigations determine possible ecological impacts owing to chemicals or other stressors based on multiple lines of evidence (Chapman, 2007). The classical Sediment Quality Triad (SQT) consists of sediment chemical analysis, examination of the in situ benthic community, and measurements of sediment toxicity (Borgmann et al., 2001). The overall study of these three components provides an assessment of the environmental risk. The SQT approach, accepted internationally as the most comprehensive approach available for assessing contaminated sediments (Chapman and McDonald, 2005), forms part of the WOE framework and is expected to be an

integral component of larger-scale assessments (Chapman and Hollert, 2006). This method has been successfully used to assess sediment quality following contaminant spill episodes (DelValls and Chapman, 1998; Chapman, 2000; Borgmann et al., 2001; Riba et al., 2004; Lee et al., 2006). In the present study this methodology was performed in order to determine whether the WOE approach is able to be used as a good tool in assessing sediment quality following the acute impact of an accidental oil spill. In addition the suitability of the application of the WOE procedure as a monitoring instrument in environmental risk assessment is demonstrated.

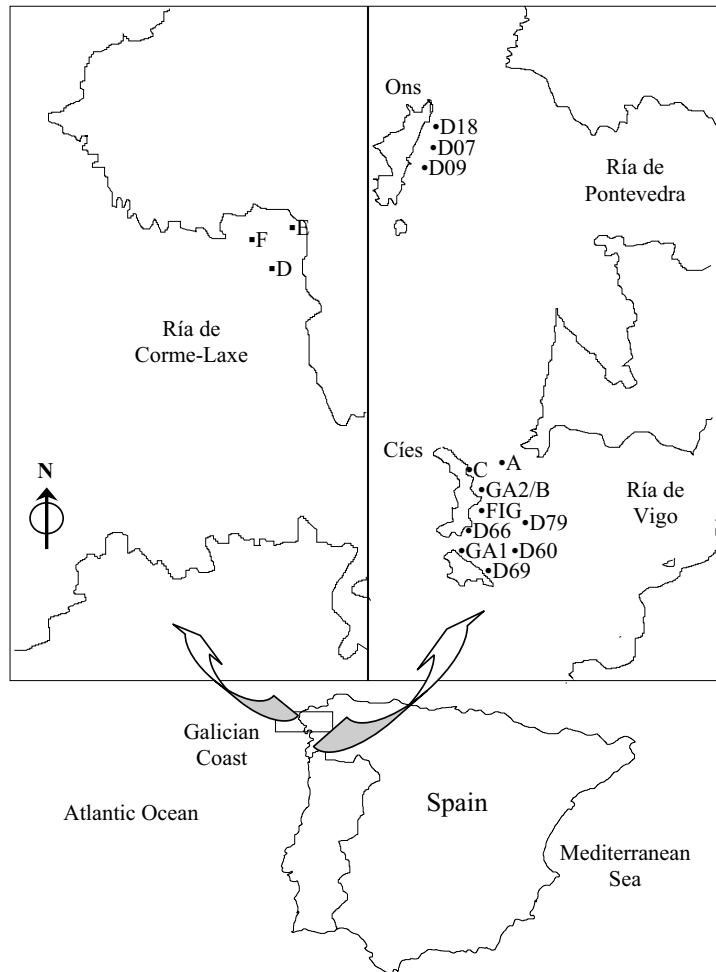
The broad aim of this study it is to determine the quality of oil spill affected sediments by applying a complete methodology, and more specifically to address the following 3 objectives: (a) to monitor the impact of an accidental oil spill using a WOE approach during a 4 year period, (b) to improve methodological aspects in the integration of the 3-LOE results in order to avoid subjectivity thereby defining a new and more objective process of integration, and (c) the determination of pollution, contamination and no impact scenarios for the stations selected and over the 4 year period, including the identification of the contaminants responsible.

## **2. Material and methods**

### **2.1. Approach**

The case study employed for the improvement of the WOE approach, was that of the impact associated with the sinking of the tanker *Prestige* (November 2002) which spilt around 60,000 tons of heavy fuel-oil with the most affected area being the Galician Coast (NW Spain). A first study was carried out with sediment samples collected in the Atlantic Islands National Park (AINP)

during 2004, approximately one year after the spill. Figure 1 shows the area of study and the 10 stations selected for the first survey described in the present paper, 3 stations located in the Ons Island (D07, D09 and D18) and 7 in the Cies Island (D60, D66, D69, D79, FIG, GA1, GA2).



**Figure 1.** Map of the coastal area of Galicia (NW Spain) showing the sampling sites in the area of Corme-Laxe (D, E and F) and the Atlantic Island National Park (Ons: D07, D09 and D18; Cies: GA1, GA2/B, FIG, D60, D66, D69, A and C).

Subsequently surveys were designed for monitoring sediment quality at the distinct stations located in the park and the surrounding area within the four year period. 4 stations were selected in the Cies Island (A, B, C and GA1), located in the AINP, for the period from the beginning of 2004 through to 2006 (3 surveys). The results obtained for the first study carried out in the AINP (beginning of 2004) suggested that the area and its surrounds were probably significantly affected by the spill. This necessitated the selection of a replacement area on the Galician Coast for inclusion in the sediment quality monitoring study. 3 stations (D, E, F) in Corme-Laxe (Figure 1) were selected and the same WOE approach applied over 2 years, from 2004/2005-2005/2006 (2 surveys).

The weight-of-evidence approach (WOE) conducted in this study includes three lines of evidence (LOEs) incorporating the following sampling station analyses carried out for each of the 3 distinct surveys described above: (a) sediment contamination: physicochemical characterization of the sediments by analyzing PAHs (acenaphtalene, acenaphtylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i) perylene, benzo(k)fluoranthene, chrysene, dibenzo(a,h) anthracene, fenantrene, fluoranthene, fluorene, indene (1,2,3,cd )pyrene, naphthalene, and pyrene), trace metals (Zn, Pb, Cu, Ni, Co and V), grain size and organic carbon (methodologies described in Morales-Caselles et al., 2006); (b) sediment toxicity: by determination of acute toxicity, performing bioassays with bulk sediment such as the amphipod mortality test with *Corophium volutator* (Morales-Caselles et al., 2007) and the polychaeta mortality assay (Casado-Martínez et al., in press) with *Arenicola marina* as well as two tests using sediment elutriate, these being the commercial assay Microtox® (Morales-Caselles et al., 2007) and

embryo–larval sea urchin bioassay (methodology described by Fernández et al, 2006; data obtained from Fernández et al. not published); (c) ‘in situ alteration’: Benthic alteration was selected and determined by measuring parameters in situ based on taxonomic identifications and community descriptive statistics (abundance-biomass analysis, species richness, diversity, dominance and proportions of the major taxonomic groups) (DeValls and Chapman, 1998).

## 2.2. Data integration

The integration of all LOE data obtained was performed via the two following methodologies: (a) a multivariate analysis approach based on linking all variables obtained in determining the environmental degradation of the studied ecosystems (Riba et al., 2004) and (b) a representation using pie charts using an ANOVA approach and by means of the determination of different factors (Riba et al., 2004).

The multivariate analysis was performed using principal components analysis (PCA) as the extraction procedure, a multivariate statistical technique for examining variable distributions (Riba et al., 2003). The objective of PCA is to derive a reduced number of new variables as linear combinations of the original variables. This provides a description of the structure of the data with minimum loss of information.

For the representation of the pie charts, the factors obtained from the PCA were subjected to ANOVA and Tukey tests which identified significant differences in sensitivity among stations and controls for each factor. In this sense, this new methodology improves and updates this kind of data treatment previously reported by Riba et al. (2003).

### 3. Results and discussion

Summarized results of the different surveys are shown in table 1 and table 2. A first approach to assess the impact of the oil spill on the Galician Coast was performed in the AINP a few months after the sinking of the tanker. The multivariate analysis was used in the data set by duplicate to connect and interpret results obtained from the three lines of evidence investigated. The application of the MAA allows the averaged variables (related to contamination, toxicity and alteration) to be grouped in a new set of factors. In this study physicochemical data (metals –Zn, Pb, Cu, Ni, Hg, V- and PAHs), toxicity (Microtox<sup>®</sup> test, amphipods assay, polychaetes test and sea urchin test) and alteration (number of species, species richness, diversity and proportions of molluscs, polychaetes and crustaceans) were included. These original variables can be grouped in four new factors which explain 84.0% of the original data variance (table 3). Negative values obtained in the analysis are as important as the positive values. Values associated with a particular component for which loading was 0.40 or higher were selected to interpret a group of variables. This approximates Comreys' cut-off of 0.55 (1973) corresponding to a good original variable factor association, while taking into account discontinuities in the magnitudes of loadings approximating the original variables.

**Table 1.** Summarized results of chemical analysis, the acute toxicity tests and the alteration parameters for our first study of the sediments quality in the Atlantic Islands National Park (D07, D09 and D18 are located in the Ons Island whereas Ga1, D60, D66, D69, D79, FIG and Ga2 are placed in the Cies Island). n.d.: not detected.

	Chemical analysis						Toxicity tests				Benthic alterations						
	Zn mgKg <sup>-1</sup>	Pb mgKg <sup>-1</sup>	Cu mgKg <sup>-1</sup>	Ni mgKg <sup>-1</sup>	V mgKg <sup>-1</sup>	Hg mgKg <sup>-1</sup>	PAH µgKg <sup>-1</sup>	Corophium %mortality	Arenicola %mortality	Microtox IC50	Paracentrotus %normal	species Nº	specific richness	Diversity	Molusc a %	Polychaet a %	Crustace a%
Ga1	14.78	2.9	12.8	1.71	1.0	0.01	190	15	5	1215	15.0	17	14.3	4.574	22.9	42.86	34.3
D-07	85.3	23.4	250.7	1.04	81.2	0.08	470	25	10	1367	3.3	17	20.5	3.76	23.5	41.17	17.7
D-09	106.9	27.5	159.7	11.7	116	0.07	240	25	15	1694	100.0	12	9.3	3.036	33.3	66.66	0.1
D-18	55.5	14	20.8	3.44	54	0.04	480	45	20	390	15.8	24	18.6	4.089	25.0	62.5	8.3
D-60	100.8	30.5	70.9	16.2	125	0.12	700	20	10	358	100.0	12	21.1	2.386	25.0	58.33	0.1
D-66	14	4.1	16.2	4.6	n.d.	0.06	380	60	15	486	5.0	6	15.4	1.231	33.3	50	16.7
D-69	14.7	2.73	12.8	1.71	n.d.	0.05	480	30	10	450	29.8	7	4.3	2.574	42.9	42.86	14.3
D-79	113.9	29.3	149.4	4.44	13.7	0.09	270	20	15	364	8.7	8	18.6	2.552	50.0	37.5	12.5
FIG	76.2	26.3	18.5	11.8	n.d.	0.04	390	25	5	1006	4.8	3	1.8	1.5	0.1	66.66	33.3
Ga2	3.95	1.14	0.65	0.42	1.0	0.01	2120	50	50	605	80.0	2	1.2	1	0.1	100	0.1

**Table 2.** Summarized results of chemical analysis, toxicity test and benthic alteration parameters measured for the study of the sediments quality in the Cies Island 2003-2006 (first survey: GA1-1, A-1, B-1, C-1; second survey: GA1-2, A-2, B-2, C-2; third survey: GA1-3, A-3, B-3, C-3) and Corne-Laxe 2004-2006 (second survey: D-2, E-2, F-2; third survey: D-3, E-3, F-3). n.d.: not detected.

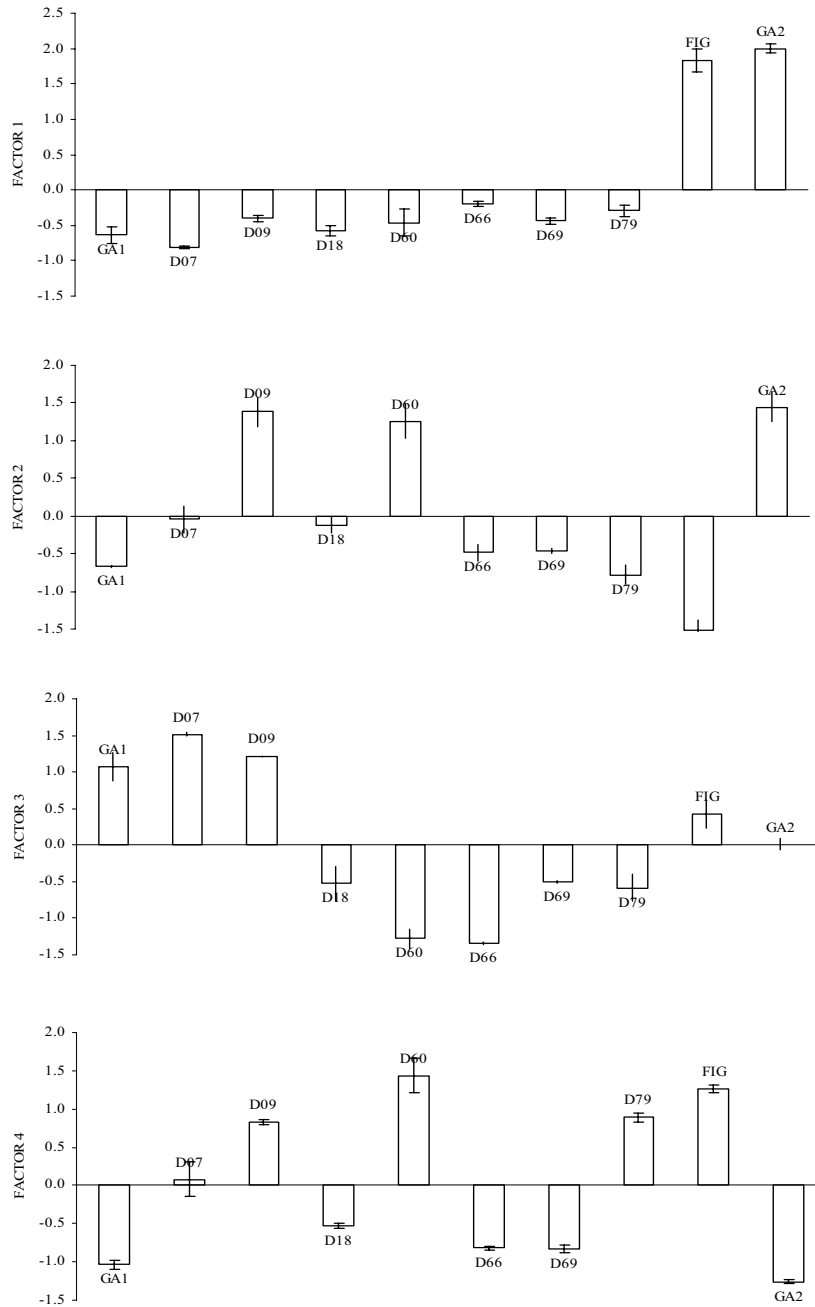
	Chemical analysis							Toxicity tests				Benthic alterations				
	Zn	Pb	Cu	Ni	V	Hg	PAH	Corophium	Arenicola	Microtox	Paracentrotus	specific richness	Diversity	Molusc a %	Polychaet a %	Crustace a %
	mgKg <sup>-1</sup>	mgKg <sup>-1</sup>	mgKg <sup>-1</sup>	mgKg <sup>-1</sup>	mgKg <sup>-1</sup>	mgKg <sup>-1</sup>	µgKg <sup>-1</sup>	%mortality	%mortality	IC50	%normal	№				
<b>GA1-1</b>	14.78	2.9	12.8	1.71	n.d.	n.d.	190	15	5	2486	85	14.3	22.9	42.9	37.1	14.3
<b>GA1-2</b>	21.2	1.6	6.4	0.32	n.d.	n.d.	74	15	5	3974	89	43.0	23.5	32.1	41.0	43.0
<b>GA1-3</b>	13.8	2.1	2.19	1.65	n.d.	n.d.	n.d.	10	0	19762	97	39.1	34.7	18.0	40.5	39.1
<b>A-1</b>	76.2	26.3	18.5	11.8	0.53	n.d.	390	25	5	1006	95	1.8	0.1	53.0	33.3	1.8
<b>A-2</b>	123.8	10.1	11.4	11.8	0.32	n.d.	119	22	10	5231	87	12.0	2.4	21.0	34.5	12.0
<b>A-3</b>	377	1.5	5.2	13.3	0.3	0.7	108	23	28	5631	79	28.5	15.3	20.0	37.0	28.5
<b>B-1</b>	3.95	1.14	0.65	0.42	n.d.	n.d.	2120	50	50	605	20	1.2	0.1	100.0	0.1	1.2
<b>B-2</b>	12.7	0.72	0.88	1.31	n.d.	1.01	366	23	35	1523	79	5.9	9.9	56.2	15.4	5.9
<b>B-3</b>	91	0.9	1.4	2.4	0.2	0.8	67	20	28	9422	88	33.9	28.4	21.5	41.0	33.9
<b>C-1</b>	41.1	7.13	24.9	5.21	0.77	n.d.	420	33	36	723	46	15.3	22.2	33.3	33.3	15.3
<b>C-2</b>	37.5	6.54	34.5	5.11	0.86	n.d.	239	28	28	4651	68	50.9	26.7	26.7	43.3	50.9
<b>C-3</b>	164	0.85	1.4	4.5	0.1	0.6	n.d.	17	22	1801	85	42.4	39.1	21.7	39.1	42.4
<b>D-2</b>	65.7	42.5	21.4	9.18	1.2	13.2	537	30	40	2436	67	25.7	33.3	33.3	33.3	25.7
<b>D-3</b>	25	3.7	0.7	1.7	0.34	2	38	10	39	3977	55	28.6	30.0	20.0	50.0	28.6
<b>E-2</b>	34	4.31	n.d.	5.66	0.38	2.33	558	36	35	20827	35	66.7	2.0	30.6	100.0	66.7
<b>E-3</b>	19.9	7.3	0.43	1.5	0.35	2.1	52	17	17	21041	85	32.1	40.1	22.2	51.4	32.1
<b>F-2</b>	214	14.6	20	7.07	0.7	5.81	820	40	40	2185	30	55.6	40.0	26.7	33.3	55.6
<b>F-3</b>	271	5.9	4.2	5.7	0.36	3.4	323	20	17	4398	76	48.2	15.4	23.1	61.5	48.2

**Table 3.** Sorted rotated factor loadings (pattern) of 17 variables for the four principal factors resulting from the multivariate analysis of the single results obtained from the chemical analysis, the acute toxicity tests and the alteration parameters for the study of the sediments quality in the Atlantic Islands National Park.

	FACTOR 1	FACTOR 2	FACTOR 3	FACTOR 4
	42.21	23.43	10.64	7.73
Zn	—	—	—	0.91
Pb	—	—	—	0.81
Cu	—	—	0.47	0.47
Ni	—	—	—	0.80
V	—	0.63	—	0.55
Hg	—	0.32	—	0.73
PAHs	0.65	0.54	—	—
Corophium bioassay	0.30	—	—	—
Arenicola bioassay	0.54	0.59	—	—
Microtox test	—	—	—	—
Paracentrotus assay	—	0.90	—	—
Number of species	0.89	—	—	—
Specific richness	0.94	—	—	—
Diversity	0.79	—	—	—
% Mollusca	0.96	—	—	—
% Polychaeta	0.77	0.48	—	—
% Crustacea	—	0.91	—	—

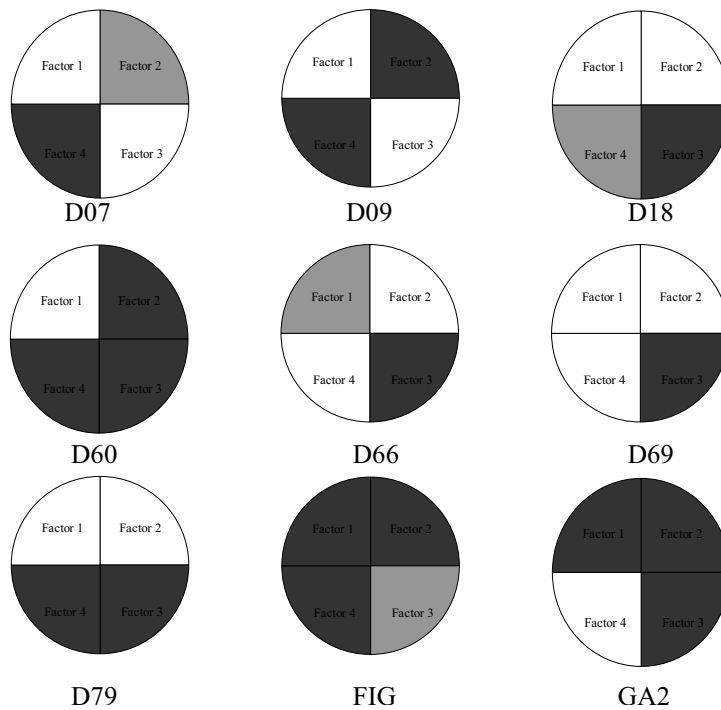
The first principal factor, #1 is predominant and accounts for 42.2% of the variance. This factor represents the degradation of the environment associated with the presence of PAHs in the sediment by linking PAHs with the toxicity of *Corphium* and the polychaete *Arenicola marina* as well as infauna alterations. The second factor explains 23.4% of the variance; showing the relationship between the presence of PAHs and the metals Hg and V in the sediment with the adverse effects measured in both the *Arenicola* and sea urchin tests and a slight alteration in the crustaceans and polychaete community. These two factors (#1 and #2), certainly appear as a consequence of the tanker *Prestige* which spilt hydrocarbons containing PAHs and affected the biota resulting in environmental degradation although Factor #2 represents more moderate effects than Factor #1; V is associated with the spill and has an apparently minor effect on biota when compared to PAHs. Factor #3 accounts for a 10.6% of the variance and is related to Cu contamination which is not responsible for biological effects in the environment, whereas factor #4 (7.7%) describes the presence of metal contamination (Zn, Pb, Cu, Ni, V and Hg) which does not produce toxic effects and is not producing degradation in the environment. This metal contamination may be related to basal levels of contaminants or owing to sources other than the *Prestige* oil spill

Figure 2 shows the factors' scores for each of the 10 studied stations. Factor #1 defined as representing the environmental degradation caused by the PAHs bound to the sediments is prevalent in the stations FIG (1.8) and GA2 (2.0) indicating high pollution levels due to PAHs in these sites. Factor #2 which indicates pollution by PAHs and V is prevalent in the station D09 (1.4) from the Ons island and D60 (1.2) and GA2 (1.4) from Cies. Factor #3, defined as representing Cu contamination with no effects has a positive loading in the samples from Ons D07 (1.5) and D09 (1.2) and FIG (0.4), GA1 (0.9) in Cies,



**Figure 2.** Estimated factor scores for the four factors in each of the 10 cases. The factor scores quantify the prevalence of each factor for every station and is used to establish the definition of each factor.

whereas Factor #4 describes the presence of metals (Zn, Pb, Cu, Ni, V and Hg) in the sediments from Ons D07 (0.1) and D09 (0.8), Cies D60 (1.4), D79 (0.8) and FIG (1.2). These results show how in 2004 the Atlantic Islands National Park was significantly affected by the oil spill and the way in which station GA1 has proved to be a suitable site of reference for the present research.



**Figure 3.** Pie charts representing the significant differences of the factors score in every study site –Atlantic Islands National Park (2003)- related to the reference site GA1 (black:  $p < 0.01$ ; grey:  $0.01 < p < 0.05$ ; white: not significantly differences,  $p > 0.05$ ). Factor #1: PAHs-pollution; Factor #2: PAHs-Hg-V-pollution; Factor #3: Cu-contamination; Factor #4: Zn-Pb-Cu-Ni-V-Hg-contamination.

With the aim of identifying the cause of pollution (or lack thereof) in each study site, an ANOVA analysis was conducted by using the factor scores obtained in the MAA. Figure 3 shows the pie charts stemming from ANOVA results, using GA1 as the negative control.

In this first approach in which the sediment status was established (in 2004) following the spill, significant pollution caused by PAHs at the stations FIG and GA2 was detected. Pollution provoked by a mixture of PAHs and V affecting both the Ons: D09 and the Cies Islands: D60, FIG, GA2 was also detected. High levels of pollution were especially identified in the station GA2 on the Cies island. A source of metals (Zn, Pb, Cu, Ni, Hg and V) was identified initially, whose presence was thought not to be in connection with the oil spills but instead is probably related to background levels or contamination which does not cause biological effects. Such levels appear at D07, D09 and the stations from Cies Islands D60, D79 and FIG. In FIG the presence of metals has been correlated with alteration in one of the analyses, but no toxicity was exhibited; perhaps meaning that alteration of the macrofauna was caused by other sources, possibly physical, such as the assessed beach cleaning after the spill. Previous studies have demonstrated that the *Prestige* oil spill caused Zn contamination in the surrounding water column (Prego and Cobelo-García, 2003). Contamination by copper and lead was also observed in the uppermost layer in the shipwreck area of the Northeast Atlantic Ocean (Prego and Cobelo-García, 2004; Cobelo-Garcia et al., 2004), however, this contamination with Cu is not likely to be related because levels of Cu in the fuel oil were relatively low ( $3.39 \text{ mg Kg}^{-1}$ ) suggesting Cu inputs from the nearby Ria de Vigo. Previous studies have shown the presence of trace metal contamination in the Rias, close to the AINP (Carballeira et al., 1997; Pérez-López et al., 2003) this also possibly explaining the presence of metals on the Cies and Ons Islands.

The application of the WOE approach has shown that some months after the spill there was a significant impact which provoked degradation of the ecosystem in part of the sediment from study sites located in the Atlantic Islands National Park. A source of metals, which in some cases are affecting the environment or are considered a potential risk has been detected. Results suggested that further studies should be done in order to clarify whether the affected AINP and surrounding sites have recovered. With this aim in mind, the WOE investigations have been applied to selected sites in the Cies Island (AINP) and Corme-Laxe, with the fresh approach of monitoring over a four year period in order to assess the recovery of an area affected by an oil spill.

Sediments from GA1 turned out to be the cleanest given it did not present toxicity or *in situ* alteration making this station an appropriate selection as the reference site in the following assessments. The WOE-monitoring focused on two areas with the following procedures applied separately: (a) assessing the Cies Island sediment quality monitoring from the beginning of 2004 to 2006, and (b) studying the Corme-Laxe sediment status from the end of 2004 to 2006. In this sense, this study was designed to monitor the recovery or persistence of the pollution caused by the oil spill over time using an improved WOE approach based on the classical SQT.

### 3.1. Cies Island (2004-2006)

The sediment quality assessment at the Cies Island (AINP) has been carried out for the same 3 sites in distinct sampling campaigns from 2003 to 2006. Results from the first survey (2004) correspond to GA1-1, A-1, B-1 and C-1; data from the second survey (2004-2005) are referred to as GA1-2, A-2, B-2 and C-2, with the results obtained in the third survey (2005-2006) corresponding to GA1-3, A-3, B-3 and C-3. The MAA was carried out by treating each set of

data as an independent case in order to track the monitoring of the sediment quality in each station.

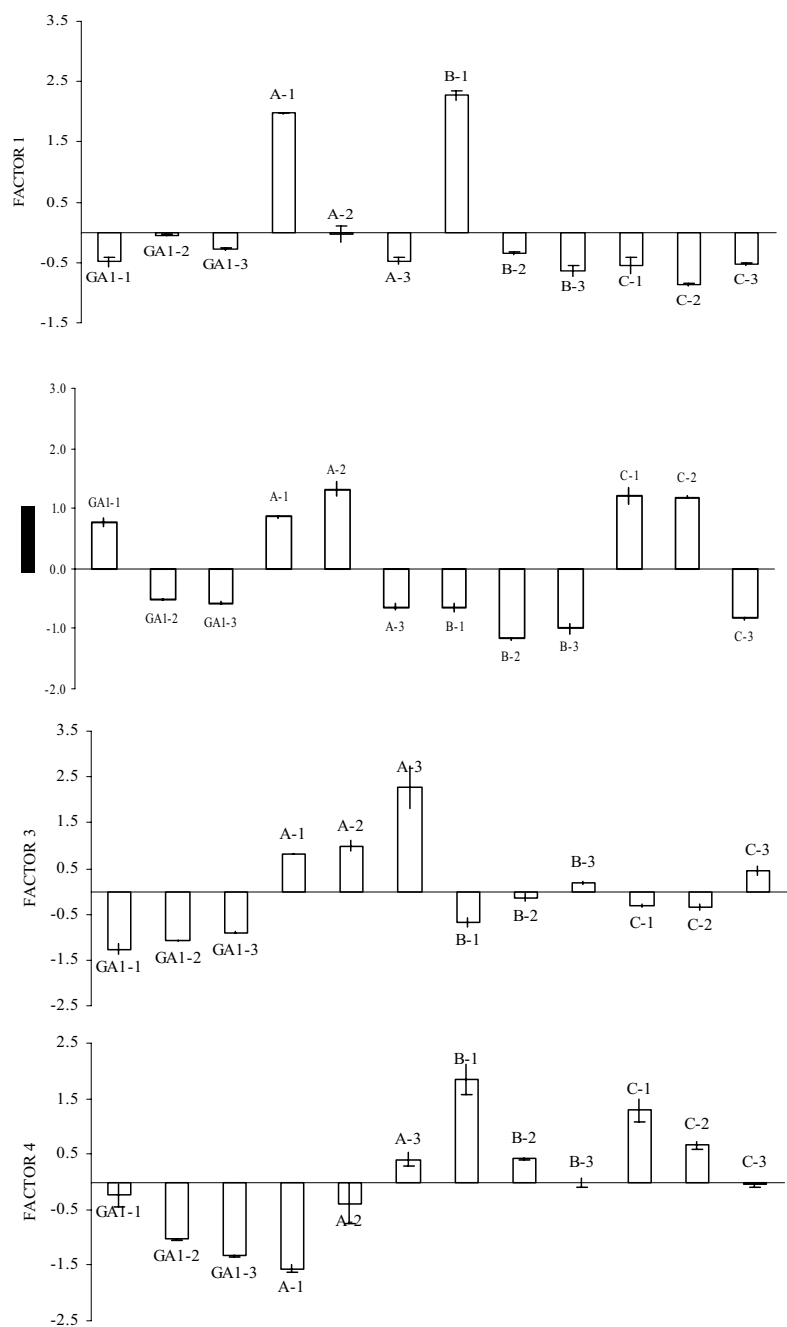
In table 4 the MAA carried out on original variable data, including replicates is shown. The application of the statistical analysis shows that the 19 original variables can be grouped in four new factors. These factors explain 88.5% of the original data variance. The first Factor (#1) accounts for 50.75 % of the variance and corresponds to the toxicity and *in situ* alteration due to PAHs and the presence of Pb responsible for environmental degradation (pollution); Factor #2 (18.3%) depicts Pb, Cu, Ni and V contamination for which no toxicity or other effects on biota is appreciable; Factor #3 (10.9%) is also related to contamination by Zn, Pb, Ni and Hg having no associated biological effects while Factor #4 (8.6%) shows a degree of toxicity and environmental degradation due to PAHs contamination.

Figure 4 shows the factor scores in the 12 cases. Factor #1, which defines pollution due to PAHs and Pb, has a positive loading in A-1 (2.0) and B-1 (2.2). These 2 cases correspond to first sampling carried out in the Cies Islands. We can see how the score for factor #1 at these 2 stations (A and B) decreases with time in the following surveys, in response to sediment recovery from the effects of initial pollution levels in the studied sites . Factor #2 and #3 demonstrate contamination by metals which was not associated with degradation in most of the stations. Factor #4, related to PAH toxicity, decreases in the stations B and C with time although slight persistence is evident in station A.

**Table 4.** Sorted rotated factor loadings (pattern) of 17 variables for the four principal factors resulting from the multivariate analysis of the single results obtained from the chemical analysis, the acute toxicity tests and the alteration parameters for the study of the sediments quality in the Cies Island 2004-2006.

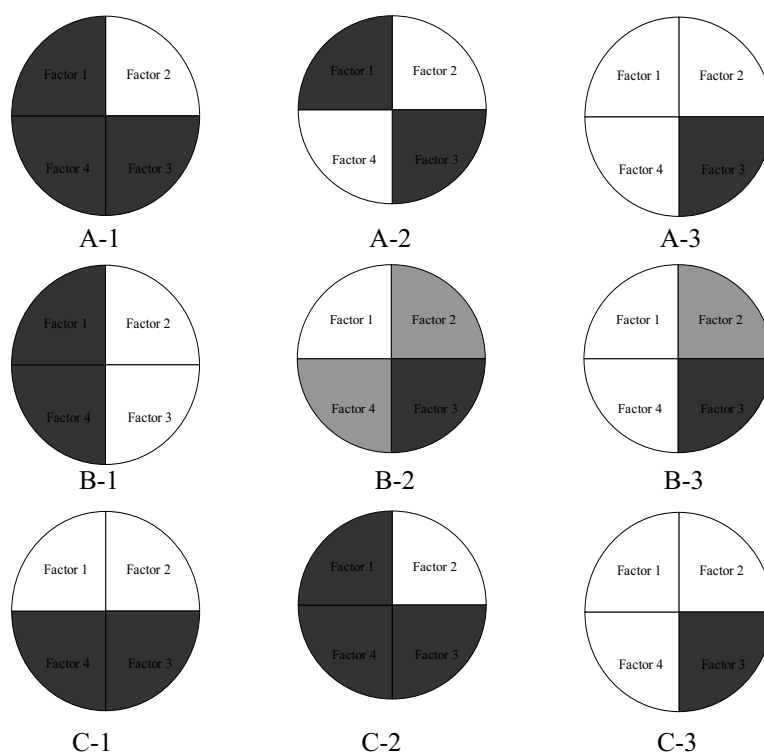
	Factor 1 50.73	Factor 2 18.26	Factor 3 10.93	Factor 4 8.62
Zn	—	—	0.88	—
Pb	0.41	0.71	0.32	—
Cu	—	0.83	—	—
Ni	—	0.43	0.86	—
V	—	0.82	—	—
Hg	—	—	0.37	—
PAH	0.73	—	—	0.63
Corophium bioassay	0.63	—	—	0.72
Arenicola bioassay	—	—	—	0.92
Microtox test	0.62	—	—	0.53
Paracentrotus assay	0.38	—	—	0.89
Number of species	0.91	—	—	0.31
Specific richness	0.95	—	—	—
Diversity	0.91	—	—	—
% Molusca	0.97	—	—	—
% Polychaeta	0.76	—	—	—
% Crustacea	0.70	—	—	0.57

Figure 5 shows the prevalence of each factor in every station according to the statistical differences obtained in the ANOVA analysis, in comparison with the reference site. The prevalence of Factor #1 and Factor #4 which are related to PAHs pollution decreased in all stations over the period 2004-2006 giving the impression that the AINP has been undergoing a process of recovery during the 4 years following the oil spill. On the other hand the significant differences found for Factor #1 and Factor #2 did not reflect such a recovery process during this period. This is perhaps related to the persistence of metal contamination in the 3 study sites, suggesting that this contamination was present prior to the spill in the studied area.



O

**Figure 4.** Estimated factor scores for the four factors in each of the 12 cases



**Figure 5.** Pie charts showing the significant differences of the factors score in every study site -Cies (2003-2006)- related to the reference site GA1 (black:  $p < 0.01$ ; grey:  $0.01 < p < 0.05$ ; white: not significantly differences,  $p > 0.05$ ). Factor #1: PAHs-Pb-pollution; Factor #2: Pb-Cu-Ni-V-contamination; Factor #3: Zn-Pb-Ni-Hg-contamination; Factor #4: PAHs-pollution.

On the whole, the analysis performed in the Cies Island for the period 2004-2006 has shown an important decrease of the initial degradation provoked by the accidental oil spill. At the start of 2004 initial pollution due to PAHs bound to sediments was detected which affected the sediment quality in stations A, B and C. This contamination and its biological effects decreased in the following surveys and currently these sediments seem not to be degraded. The presence of metals contamination was detected in the stations despite this not having produced environmental biological effects. It is possible these metals may not be available to organisms in their present form, but that if environmental conditions eventually changed, they may become a threat for the environment.

### 3.2. Corme-Laxe (end of 2004-2006)

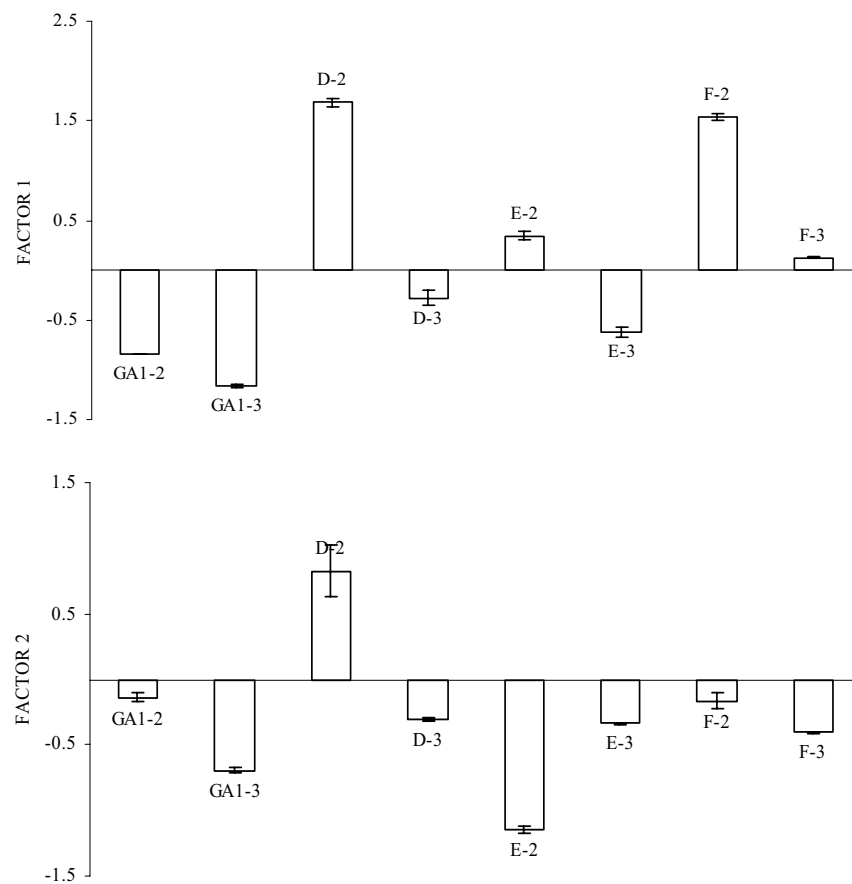
The sediment quality evaluation was performed at the same sites during distinct surveys in the period from the end of 2004 to 2006. Results from the first survey (2004-2005) correspond to GA1-2, D-2, E-2 and F-2; data from the second campaign are referred to as GA1-3, D-3, E-3 and F-3.

The multivariate approach was conducted as described above. After applying the principal factors analysis the 17 variables were grouped in two new factors (table 5). These factors explain 62.96 % of the original data variance. The first factor, #1 is predominant and explains a 41.3 % of the variance. It links the pollution caused by PAHs and metals (Zn, Pb, Cu, Ni, V and Hg) bound to sediment by relating these contaminants with the toxicity (amphipods, polychaete, sea urchin) and alteration (number of species and percentage of crustacea). The second factor, #2, explains 21.6 % of the variance and depicts the relationship between certain metals (Cu and V) with alteration (specific richness, % of polychaete) and potential toxicity (Microtox® test).

**Table 5.** Sorted rotated factor loadings (pattern) of 17 variables for the four principal factors resulting from the multivariate analysis of the single results obtained from the chemical analysis, the acute toxicity tests and the alteration parameters for the study of the sediments quality in Corme-Laxe 2004-2006.

	FACTOR 1	FACTOR 2
	41.32	21.64
Zn	0.55	—
Pb	0.79	—
Cu	0.70	0.59
Ni	0.94	—
V	0.64	0.72
Hg	0.88	—
PAH	0.89	—
Corophium bioassay	0.88	—
Arenicola bioassay	0.83	—
Microtox test	—	0.87
Paracentrotus assay	0.72	—
Number of species	0.50	—
Specific richness	—	0.92
Diversity	—	—
% Mollusca	—	—
% Polychaeta	—	0.78
% Crustacea	0.49	—

Figure 6 shows the factor scores for the 8 cases. Factor #1, which is defined as the pollution caused by PAHs and the metals Zn, Pb, Cu, Ni, V and Hg, has a positive loading in D-2 (1.7), E-2 (0.3), F-2 (1.5) and F-3 (0.1). The three first cases correspond to the first Corme-Laxe survey (2004-2005) whereas F-3

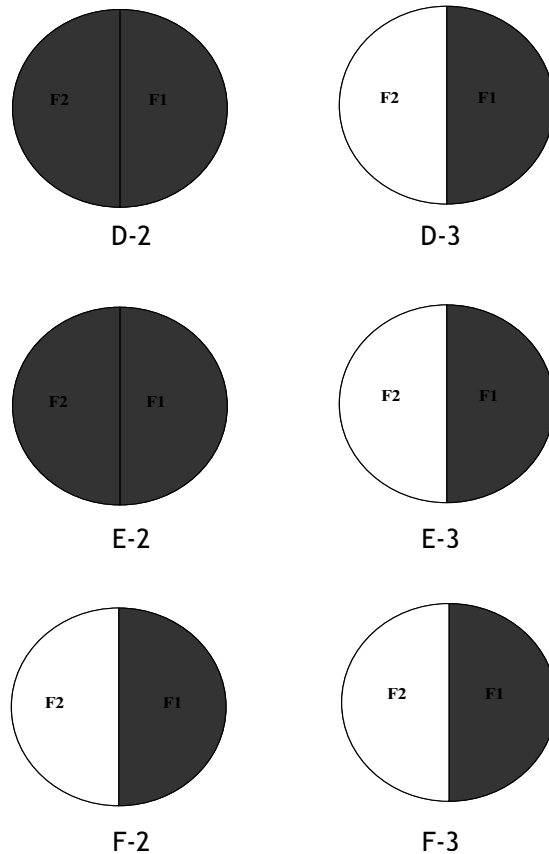


**Figure 6.** Estimated factor scores for the two factors in each of the 8 cases.

corresponds to the second station F survey (2005-2006). We can see how the score for factor 1 in D, E, and F decreased as time went on, while remaining positive for station F, this indicating that sediments from these stations have recovered but that degradation persists in station F. Factor #2 shows metal contamination by Cu and V linked to alteration and potential toxicity having a positive loading for D-2 (0.8). The factor score decreased with time in stations D and F whereas station E presented an increase despite it not having a positive loading. Figure 7 shows the prevalence of each factor in every station according to the statistical differences obtained in the ANOVA analyses in comparison with the reference site (GA1). The significant differences in Factor #1 compared with the reference station did not decrease during the 2004-2006 period with respect to the stations D, E and F, meaning that although recovery was detected in the MAA analysis, D and E are not as clean as the reference station, whereas site F continued to present degradation due to a mixture of various contaminants including PAHs and metals. On the other hand the potential pollution due to the metals Cu and V (Factor #2) present in D and E, was not apparent for the final survey, insofar as significant differences compared with the control GA1 were concerned.

The Corme-Laxe study showed that the presence of both PAHs and a mixture of metals Zn, Pb, Cu, Ni, V and Hg initially caused environmental degradation at the stations D, E and F. The recovery of the stations in D and E (MAA) show that the source of the PAHs pollution is related to the *Prestige* oil spill. However, the presence of metals with different characteristics from those bound to the original fuel oil from the *Prestige*, suggest the possible existence of another source or sources of contamination in the area. Previous studies have shown that the neighbouring Rias and coastal waters act as a source of dissolved and particulate trace metals (Cobelo-García et al., 2005). Research

results obtained show sediments from the stations D and E to have recovered whereas degradation remains at the F site nearest to the coast, highlighting the influence of the mentioned causes different than the Prestige. Initial potential degradation caused by Cu and V was also detected, although not present for the final survey.



**Figure 6.** Pie charts showing the significant differences of the factors score in every study site –Corme-Laxe (2004-2006)- related to the reference site GA1 (black:  $p < 0.01$ ; grey:  $0.01 < p < 0.05$ ; white: not significantly differences  $p > 0.05$ ). Factor #1: PAHs-Zn- Pb-Ni-Cu-V-Hg-pollution; Factor #2: Cu-V-pollution.

## 4. Conclusions

The WOE approach employed in this study has been applied to 3 lines of evidence (contamination, toxicity and alteration) in addressing 3 distinct objectives. First of all a revision of the sediment quality following the *Prestige* oil spill has been carried out in the Atlantic Islands, an area with a high ecological relevance. This was achieved by applying a multivariate and ANOVA analyses. In order to assess the development of the quality of the sediments affected by the spill a set of stations was studied using the same time-dependent methodology for the Cies Island (2004-2006) and in Corme-Laxe (2004-2006). Results obtained have identified PAHs related to the *Prestige* oil spill as the main contaminant in the sites studied on the Galician Coast. A source of metals has been identified in the Atlantic Islands National Park which seems not to be producing biological effects although further research of this input of metals should be carried out especially for the Ons Island. In Corme-Laxe an additional source or sources of a mixture of contaminants was also detected. Pollution has decreased in recent years in both the Atlantic Islands National Park and Corme-Laxe areas, although there is still some degradation present in some areas, particularly in Corme-Laxe.

The information obtained in this study has demonstrated that WOE is a suitable tool for monitoring environmental risk assessment allowing sources and fate of contaminants to be differentiated in addition to their potential risk. The innovative application of the classical WOE methodology has proved useful in obtaining more objective results and its use is recommended in the design and implementation of monitoring programs in areas that have suffered contamination episodes through the selecting of appropriate lines of evidence on a case by case basis

## 5. Acknowledgements

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# **A weight of evidence approach for quality assessment in sediments impacted by an oil spill: the role of a new line of evidence using a set of biomarkers**

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## **Abstract**

A set of biomarkers has been chosen and analyzed in target tissues of two invertebrate species after a 28-d exposure to sediments from the Galician Coast in an attempt to incorporate a new line of evidence (LOE) to a classical weight of evidence (WOE), an approach designed to assess sediment quality four years after the oil spill of the tanker *Prestige* (2002). Sublethal bioassays with crabs and clams were carried out under laboratory and field conditions and included the determination of: Ethoxyresorufin O-deethylase (EROD) activity to assess the phase I detoxification system; glutathione-S-transferase (GST) as a phase II detoxification enzyme but also implicated in oxidative stress events; glutathione peroxidase (GPX), glutathione reductase (GR) and the ferric reducing ability of plasma (FRAP) assay were analyzed to determine the tissues' antioxidant activity. The integration of biomarkers with sediments contamination, acute toxicity and benthic alteration parameters provide an "early warning" tool which not only indicates the environmental quality of an area, it also constitutes an advisory tool for potential ecological risks. The present study demonstrates that the use of the set of biomarkers as part of a WOE approach designed to assess contaminated sediments contributes added

value to the classical LOEs and allows characterizing the environmental status of the studied area in a more precise and accurate way.

*Keywords: PAHs, contamination, toxicity, sublethal, WOE.*

## **1. Introduction**

Chemical analysis normally is the main tool in sediment quality assessment even though chemical concentrations alone are inadequate for prediction of biological consequences. The biological effects can be established based on laboratory tests that determine toxic responses, as well as field data on the communities living in the sediments allow to establish whether there is observable pollution-induced degradation effect in the biota [1]. Weight of evidence (WOE) investigations determines possible ecological impacts from chemicals or other stressors based on multiple lines of evidence (LOEs) [2] and have been widely used in recent years to assess sediment quality around the world [3,4] including different areas in the Iberian Peninsula [5,6,7,8,9].

Since the sinking of the tanker *Prestige* (2002), which spilt about 63,000 tonnes of heavy fuel oil (a mixture of saturated hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes, with most of the PAHs being of medium to high molecular weight) and mainly affected the Galician Coast, several investigations have focused on determining the biological effects and environmental status after this dramatic episode by following single lines of evidence, such as chemical analyses [10,11,12], toxicity [13,14,15,16] or benthic alteration [17,18]. Recently, authors presented a report [9] where a classical WOE approach based on three lines of evidence (physicochemical characterization of the sediments, determination of acute toxicity and benthic alteration) was carried out in the Galician Coast; the sediment quality of the area was monitored during the time and a general recovery of the

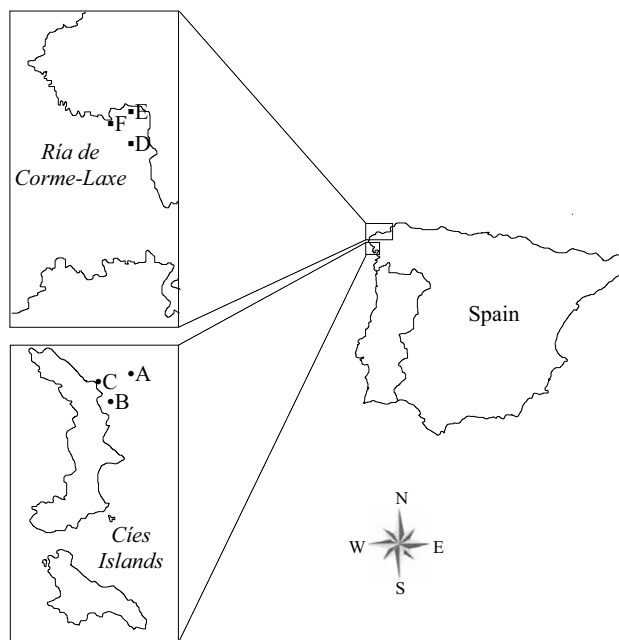
environmental health was observed. However there were signs that other sources of contaminants apart from the *Prestige* oil spill could be producing some environmental stress to the exposed organisms. The aim of the present study is to use a new line of evidence (LOE) with the weight of evidence (WOE) approach to improve the sediment quality assessment conducted by previous studies in the Galician Coast. The lines of evidence selected constitute an improvement of the classical WOE including laboratory and field studies based on biomarkers determinations. Biomarkers can act as an important early warning system by telling whether environmental pollutants are present at sufficiently high concentrations to cause an effect [19]. Chemicals such as the Polycyclic Aromatic Hydrocarbons (PAHs) have very short biological half-lives in most species but may nevertheless have long-term effects [19]. In this sense, some compounds might not produce acute toxic effects but sublethal effects can be expected. The main aims of this research are: (a) to prove the feasibility and viability of incorporating new lines of evidence to the classical methodology employed in the WOE and its application to assess the environmental quality of oil contaminated sediments , (b) to monitor the sediment quality 4 years after the impact of an accidental oil spill by using a newly WOE approach, (c) to determine the extent of the impact from the spill addressing the contamination, pollution and no effects in the stations selected, including the identification of the contaminants responsible for the damage.

## **2. Material and methods**

### **2.1. Approach**

The study was performed on two areas of the Galician Coast (NW Spain) importantly affected by the *Prestige* oil spill in 2002 (Figure 1): Cies Island (A, B, C) in the Atlantic Island National Park and the Bay of Corme-Laxe (D, E, F).

Cies Island, located in the Atlantic Island National Park acted as a natural barrier protecting the rias from the entrance of the fuel. The Bay of Corme-Laxe is also considered a place with high ecological relevance with a low anthropogenic and industrial influence with fishing and farming being the main economic activities.



**Figure 1.** Map of the coastal area of Galicia (NW Spain) showing the sampling sites in the Atlantic Island National Park (A, B, C) and the area of Corme-Laxe (D, E and F).

The 4 lines of evidence employed in the WOE approach included:

(a) sediment contamination: physicochemical characterization of sediments by analyzing PAHs (acenaphtalene, acenaphtylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i) perylene, benzo(k)fluoranthene, chrysene, dibenzo(a,h) anthracene, fenantrene, fluoranthene, fluorene, indene (1,2,3,cd )pyrene, naphthalene, and

pyrene) using GC-MS with selected ion monitoring and trace metals (Zn, Pb, Cu, Ni and Hg) with anodic voltamperimetry [16];

(b) acute toxicity and bioaccumulation: by performing sediment bioassays such as the commercial assay Microtox® [20], the amphipod mortality test with *Corophium volutator* [20], the polychaeta mortality assay [21] and bioaccumulation experiment with *Arenicola marina* [22];

(c) 'in situ alteration': Benthic alteration was selected and determined by measuring parameters in situ based in taxonomic identifications and community descriptive statistics (abundance-biomass analysis, species richness, diversity, dominance and proportions of the major taxonomic groups) [5];

(d) laboratory and field studies based on biomarkers by using two invertebrate species, the crab *Carcinus maenas* and the clam *Ruditapes philippinarum*, and a set of biomarkers [23,24]: mixed function oxygenase activity, which is the first mode of detoxification in many organic pollutants, was measured using the adapted EROD assay; the phase II metabolizing enzyme Glutathione-S-transferase (GST) activity was determined by monitoring the rate of conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm; the oxidation of 1 mM NADPH by Glutathione Reductase activity (GR) in the presence of 10 mM oxidized glutathione was also monitored at 340 nm; the antioxidant Glutathione Peroxidase activity (GPX) was analyzed by determining the oxidation of NADPH with the presence of 1.25 mM hydrogen peroxide; the FRAP assay, ferric reducing ability of plasma, allows a measure of the antioxidant capacity; all the biomarkers responses were normalized with the total protein content.

## 2.2. Data integration

The data obtained from the different LOEs were integrated through a multivariate analysis approach based on linking all the variables obtained [7] and a pie chart representation of comparisons between sites of multivariate factors [7,9]. The multivariate analysis was performed using principal component analysis (PCA) as the extraction procedure, which is a multivariate statistical technique to explore variable distributions [25]. The original data set used in the analysis included the variables obtained from the 4-LOEs and its objective was to derive a reduced number of new variables as linear combinations of the original variables. This provides a description of the structure of the data with the minimum loss of information. For the representation of the pie charts, the new factors obtained from the PCA were submitted to ANOVA and Tukey tests which identified significant differences in sensitivity among stations and controls for each factor [9]; every study site has a pie chart divided into the obtained factors which use different colours depending on the level of significant differences in relation with the reference.

### **3. Results and discussion**

Table 1 shows the summarized results of the different parameters analyzed. In general, the concentration of chemicals varies among the stations from the AINP (A, B and C) and those from Corme-Laxe (D, E, and F) although no general pattern was observed, except for Hg which was higher in all Corme-Laxe sites. Station A and F presented the highest contents in metals. Mainly, acute toxicity and PAHs bioaccumulation was higher in organisms exposed to sediments from the Bay of Corme-Laxe whereas biomarkers responses were also higher in the area of Corme-Laxe, both under field and laboratory deployments. It was not observed a general pattern in benthic parameters between the sampling sites.

**Table 1.** Summarized results of chemical analysis (mgKg<sup>-1</sup> for metals, µgKg<sup>-1</sup> for PAHs) the acute toxicity tests (Corophium and Arenicola: % mortality; Microtox: IC50; bioaccumulation of PAHs: µgKg<sup>-1</sup>), biomarker responses under field and laboratory conditions (glutathione peroxidase activity GPX: nmol/min/mg prot, glutathione transferase GST activity nmol/min/mg prot, glutathione reductase GR activity nmol/min/mg prot, ferric reducing ability of plasma FRAP activity µM/mg/min and EROD activity pmol/mg/min) and the alteration parameters for sediments from the AINP (A, B, C) and Corne-Laxe (D, E, F). n.d.: not detected; n.a.: not available.

		<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>E</b>	<b>F</b>
<b>Chemical analyses</b>	<b>Zn</b>	377	91	164	25	19.9	271
	<b>Pb</b>	1.5	0.9	0.85	3.7	7.3	5.9
	<b>Cu</b>	5.2	1.4	1.4	0.7	0.43	4.2
	<b>Ni</b>	13.3	2.4	4.5	1.7	1.5	5.7
	<b>Hg</b>	0.7	0.8	0.6	2	2.1	3.4
	<b>PAH</b>	108	67	n.d.	38	52	323
<b>Toxicity tests</b>	<b>Corophium</b>	23	20	17	10	17	20
	<b>Arenicola</b>	28	28	22	39	17	17
	<b>Microtox</b>	5631	9422	1801	3977	21041	4398
	<b>Bioaccumulation</b>						
	<b>PAH</b>	2927	2573	2666	2616	3912	3285
<b>Biomarkers (laboratory)</b>	<b>GPX-crab-lab</b>	11.6	9.7	8.2	19.3	19.5	15.9
	<b>GPX-clam-lab</b>	2.1	2.9	4.5	6.1	3.1	4.2
	<b>GR-crab-lab</b>	1.1	0.7	0.9	0.9	0.6	1.5
	<b>GR-clam-lab</b>	2.1	1.6	2.3	3.4	11.7	4.0
	<b>GST-crab-lab</b>	140	218	407	430	684	1071
	<b>GST-clam-lab</b>	1293	839	1624	1199	910	848
	<b>EROD-crab-lab</b>	0.1	0.1	0.1	0.0	0.1	0.1
	<b>EROD-clam-lab</b>	0.3	0.3	0.4	0.4	0.4	0.2
	<b>FRAP-crab-lab</b>	3.9	2.1	2.6	2.9	2.9	1.6
	<b>FRAP-clam-lab</b>	10.6	7.8	4.0	13.7	12.1	6.4
<b>Biomarkers (field)</b>	<b>GPX-crab-field</b>	17.8	23.1	15.9	41.4	193.1	125.7
	<b>GPX-clam-field</b>	10.5	3.6	4.0	25.5	3.2	7.0
	<b>GR-crab-field</b>	0.7	1.4	1.4	9.9	9.5	23.4
	<b>GR-clam-field</b>	2.9	1.3	3.8	9.7	14.7	8.0
	<b>GST-crab-field</b>	1098	1564	690	1489	7523	6073
	<b>GST-clam-field</b>	2061	372	1199	3366	131	1558
	<b>EROD-crab-field</b>	0.1	3.0	0.0	8.5	0.4	0.5
	<b>EROD-clam-field</b>	0.2	0.1	0.1	0.6	0.1	0.1
	<b>FRAP-crab-field</b>	2.7	n.a.	n.a.	2.4	n.a.	n.a.
	<b>FRAP-clam-field</b>	10.4	3.1	2.6	23.6	2.0	6.6
<b>Benthic alterations</b>	<b>Number of species</b>	28.5	33.9	42.4	28.6	32.1	48.2
	<b>specific richness</b>	5.1	5	4.3	3	3	2.9
	<b>Diversity</b>	15.3	28.4	39.1	30	40.1	15.4
	<b>Dominance</b>	0.50	0.10	0.06	0.15	0.19	0.20
	<b>% Mollusca</b>	15.3	28.4	39.1	30.0	32.4	15.4
	<b>% Polychaete</b>	20.0	21.5	21.7	20.0	20.4	23.1
	<b>% Crustacea</b>	37.0	41.0	39.1	50.0	47.2	61.5

The multivariate analysis was used in the original data set by using replicates (not averages) in order to link the results obtained from the different lines of evidence investigated. The factor analysis reveals that the original variables can be grouped into three new factors which explain a 79 % of the total variance (Table 2). The multivariate analysis is a tool that allows us to interpret a large group of different variables by grouping them using correlations; in addition it indicates the importance of each factor in every single study site.

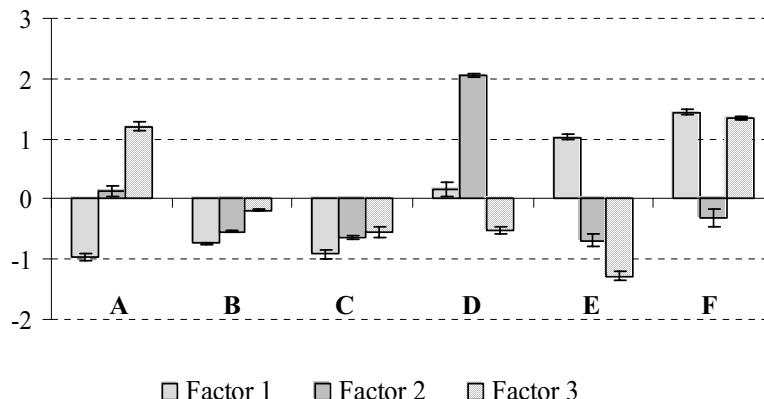
**Table 2.** Sorted rotated factor loadings of 36 variables for the three principal factors resulting from the multivariate analysis of the single results obtained from the chemical analysis, the acute toxicity tests, the suite of biomarkers and the alteration parameters for the study of the sediments quality in the Galician Coast. Chemicals: loadings are related to the concentration of contaminants in sediments; Acute effects: loadings explain the toxicity detected by the acute assays and the bioaccumulation of PAHs in *Arenicola*. Sublethal effects: loadings are related to the induction of biomarkers. Benthic alteration: loadings are related to alteration of the biota (decrease of number of species, specific richness, diversity, diminution in the percentage of molluscs and crustacea and increase on the polychaete population. The group of variables selected for the interpretation presented a loading 0.40 or higher for a good association between an original variable and a factor.

		<b>Factor 1</b> 35.2	<b>Factor 2</b> 24.5	<b>Factor 3</b> 19.2
<b>Chemicals</b>	Zn	—	—	0.90
	Pb	0.95	—	—
	Cu	—	—	0.93
	Ni	—	—	0.76
	Hg	0.95	—	—
	PAH	0.59	—	0.76
<b>Acute effects</b>	Corophium	—	-0.52	0.75
	Arenicola	-0.41	0.87	—
	Microtox	—	—	—
	Bioaccumulation	0.73	—	—
<b>Sublethal effects (laboratory &amp; field)</b>	GPX-crab-lab	0.48	-0.77	—
	GPX-clam-lab	—	0.53	—
	GR-crab-lab	0.41	—	0.78
	GR-clam-lab	0.65	—	0.49
	GST-crab-lab	0.85	—	—
	GST-clam-lab	-0.53	—	—
	EROD-crab-lab	0.62	-0.78	—
	EROD-clam-lab	—	—	-0.89
	FRAP-crab-lab	-0.46	—	—
	FRAP-clam-lab	—	0.64	—
	GPX-crab-field	0.88	—	—
	GPX-clam-field	—	0.99	—
	GR-crab-field	0.91	—	—
	GR-clam-field	0.77	—	-0.46
	GST-crab-field	0.89	—	—
	GST-clam-field	—	0.75	—
	EROD-crab-field	—	0.88	—
	EROD-clam-field	—	0.98	—
	FRAP-crab-field	—	0.76	—
	FRAP-clam-field	—	0.99	—
<b>Benthic alteration</b>	Number of species	—	0.56	—
	specific richness	0.91	—	—
	Diversity	—	—	0.97
	% Mollusca	—	—	0.96
	% Polychaeta	0.62	-0.68	—
	% Crustacea	-0.96	—	—

### ***Factor #1***

The main factor, Factor 1, accounts for a 35.2 % of the variance and shows the relationship between different variables related with chemicals, sublethal responses, bioaccumulation and benthic alteration. The concentration of PAHs, Pb and Hg in sediment is related to the bioaccumulation of PAHs in *Arenicola marina* exposed under laboratory conditions, but opposite to their mortality in the acute assays. A set of antioxidant and detoxification biomarkers including GPX, GR, GST, EROD and FRAP activities analyzed in crabs under laboratory conditions are correlated in the Factor #1, in addition to GR and GST activity in the digestive gland of clams. EROD and FRAP in the field exposures were not correlated to other variables. The aforementioned contaminants and the toxicity variables are slightly connected to the benthic alteration explained by alteration of the specific richness and an increase of the polychaete population, while a positive development of crustaceans was detected. The combination of this large group of variables in Factor #1 is interpreted as a contamination by PAHs mixed with the metals Hg and Pb which are not producing lethal effects, although PAHs bioaccumulation and sub-lethal responses in organisms are generated resulting in a slight alteration of the *in situ* benthic community. Environmental alterations due to these contaminants have been reported by other authors [26]. This factor has a positive effect in the stations E (1.0) and F (1.4) located in Corme-Laxe (Figure 2). The hydrodynamics of the Bay of Corme-Laxe suggest an accumulation of contaminants including fuel oil from the *Prestige* [27, 28] what could explain the sediment contamination and effects of the study sites. Previous studies did not detect the presence of Pb and Hg concentrations in emulsified samples of the *Prestige* fuel (with 54–59% water) [29] although the Pb origin in the polluting oil was corroborated by other authors [30]. Taking this factor into account seems to

describe the pollution caused by the remaining contaminants from the fuel spill by the tanker *Prestige*, as was shown in the previous study [22].



**Figure 2.** Estimated factor scores for the three factors in each of the 6 cases. The factor score quantify the prevalence of each factor for every station and is used to establish the definition of each factor.

Despite toxicity tests did not demonstrate the acute effects of these contaminants (PAHs, Pb and Hg), the bioaccumulation of PAHs experienced by *A. marina* in the conducted bioassays demonstrate the bioavailability of these substances. The induction of different biomarkers in the hepatopancreas of crabs and in the digestive gland of clams have been related to the presence of these contaminants what suggests that the deployed organisms suffered stress due to the presence of this substances in the sediments; the correlation observed among the biomarkers and the different variables defined by Factor #1 is stronger for those enzyme activities measured in organisms exposed under laboratory conditions what imply that field deployments result in less sensitivity. Most likely, the effects of the contaminants within sediment are reduced because of the flushing action of the open water environment. In

addition, the crab *Carcinus maenas* has shown to be more perceptive than the clam *Ruditapes philippinarum* to assess this kind of pollution, although in general, a good correlation was detected among the biomarkers induced in both invertebrate species. On the other hand some of the variables related to the benthic alteration present in Factor #1 corroborate the effects observed in the sublethal experiments.

### ***Factor #2***

The second factor, Factor #2 (24.5 % of the variance) connects the set of biomarkers measured under field conditions (EROD and FRAP activity in crabs and clams, and GPX and GST in clams), the mortality of *Arenicola* in the acute experiment, no toxicity for amphipods, the alteration in the number of species and the decrease of the polychaete population. Positive and negative correlations for a few biomarkers were also detected under laboratory conditions, and no accordance with the amphipod toxicity test was observed. The relationships between the biological responses identified in Factor #2 are not correlated with any of the chemicals analyzed what suggest that a contaminant or group of compounds bound or not to the sediment which were not analyzed are the cause of the biological effects. Taking into account that the acute toxicity observed by the *Arenicola* and the rest of the bioassays was relatively low (less than 30% mortality in most of the cases) [22], a source of contaminant not related to sediment is the most probable cause of these effects. Station A (0.1) located on Cies Island and mainly the site D (2.0) in the bay of Corme-Laxe present positive loading of Factor #2 (Figure 2). The good correlation experienced by the biomarkers measured in both crabs and clams under field conditions suggest that these locations suffer the stress of non-measured variable/s which in the case of site D could be related to the proximity of aquaculture infrastructures for mussel growth. Other authors [31],

have described the negative impacts of these rafts for mussel aquaculture, including: the discharge of a large volume of bio-deposits containing high concentrations of nutrients; the release of drugs and pesticides into the environment; an increase in sedimentation and accumulation of organic matter; an increase in the concentration of nutrients in sediments and waters (mainly N and P). Negative effects on wild populations of animals have also been reflected, ranging from genetic interaction and disease transmission, to changes in the composition of the structure of benthic fauna due to a change from oxic to anoxic conditions [31].

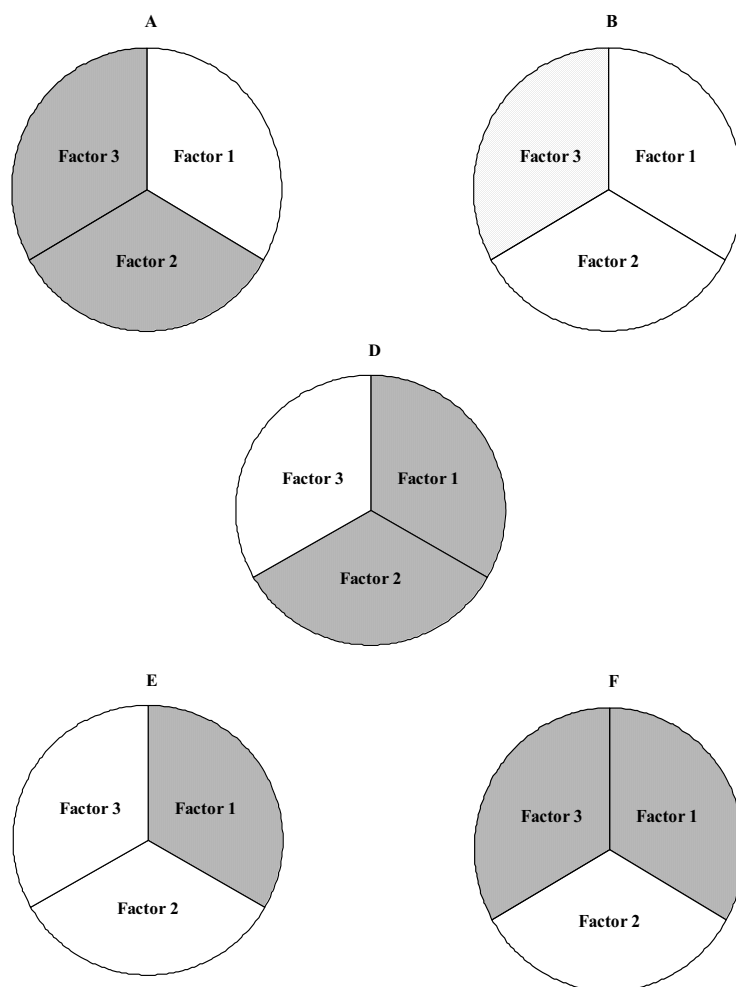
### ***Factor #3***

The third factor, Factor #3 accounts for a 19.2 % of the variance and shows the sediments contamination by the metals Zn, Cu and Ni, and the PAHs; this contaminants are related to acute toxicity determined by the amphipods assay, which is not significant (the toxicity detected was not enough to consider sediment samples as toxic according to this acute assay: samples where the mortality rate of the amphipods is 20% higher than the mortality recorded in the reference and show significantly different (\* $p < 0.05$ ) results compared to those obtained in the reference are considered as toxic) [22] and antioxidant responses under laboratory assays with crabs and clams (GR); the effects on the benthic community are shown as an alteration of the diversity and percentage of molluscs. These correlations suggest the presence of some stress in the environment due to a source or sources of metals (Zn, Cu and Ni) and organic compounds (PAHs) different to the source explained by Factor #1. In this case, Factor #3 presents a positive effect in sites A (1.2) in the AINP and F (1.3) in the Bay of Corme-Laxe (Figure 2). Previous studies in the area of Corme-Laxe have detected severe contamination by Cu in the sediments [28], however a fuel oil origin was unlikely and a major source of Cu related to antifouling

paints from the hulls of fishing vessels was suggested [32]. Although contamination by Cu and Zn was observed in the uppermost layer in the *Prestige* shipwreck area of the Northeast Atlantic Ocean [33, 34], this contamination should not be related to the shipwreck, because levels of Cu in the fuel oil carried by the *Prestige* were relatively low ( $3.39 \text{ mg kg}^{-1}$ ) and previous studies have shown that inputs from terrestrial sources of metals are probably higher than inputs from the spilled fuel oil [35, 36]. Some of the studied variables demonstrate the stress of these contaminants and the effects on the benthic community; however, the results mostly point to chronic contamination with low bioavailability, and potential but largely unconfirmed biological risk. In this sense previous studies [37] considered that despite a high percentage of the total content of trace metals in sediments from the Galician coast presented a reactivity and bioavailability were very low, the high degree of pyritization found for some of the most toxic trace metals may favour their release by oxidation of the sulphides that they form, thus making them bioavailable to benthic fauna.

#### *Significant differences among stations*

From the results obtained, the authors considered station C, which presented an absence of PAHs contamination and the lowest biological effects, as a suitable site to use as reference station. Taking this into account, the factor loadings obtained in the MAA were submitted to ANOVA and Tukey test in order to determine the significant differences between the stations and the reference site for each of the three displayed factors to identify the cause which is producing (or not) pollution in every single study site (Figure 3).



**Figure 3.** Pie charts which represent the significant differences of the factors score in every study site related to the reference site C (dotted:  $p < 0.01$ ; slightly dotted:  $p < 0.05$ ; not dotted: no significant differences,  $p > 0.05$ ).

None of the sediments from Cies Island in the AINP presented significant differences in Factor #1 which means that the effects of the *Prestige* oil spill are not still occurring in the area. However, site A presents significant differences ( $p < 0.01$ ) with the selected reference station (C) related to Factor # 2 and # 3 which suggests the potential risk and the environmental stress caused by non-measured substances coming from other sources apart from the tanker *Prestige*. Site B presents significant differences ( $p < 0.05$ ) with the reference station according to Factor #3, what means that the presence of some contaminants in the area are considered a potential risk, although in general, the sediments present a relatively good environmental quality. On the other hand, sediments from Corme-Laxe D, E and F show significant differences ( $p < 0.01$ ) with the reference station for Factor #1 meaning the remaining contaminants from the *Prestige* oil spill are still producing sub-lethal effects to the biota of the bay. In addition, the significant differences ( $p < 0.01$ ) shown for Factor #2 in D focus to other unknown sources of contaminants responsible of biological stress in the study site, whereas in the case of site F a mixture of metals and PAHs from different sources could be considered a potential risk in the area as it is shown in Factor #3 ( $p < 0.01$ ).

It is well known that biomarkers have been shown to be useful “early warning” tools in characterizing the health status of animals from impacted areas [38, 39], such as oil affected places [40], where complex mixtures of pollutants are usually present. In the present study it has been show how the use of the set of biomarkers as part of a WOE approach designed to assess contaminated sediments contributes added value to the classical LOEs original idea and allows for the characterization of the environmental status of the studied area in a more precisely and accurately way. In addition the inclusion of chronic bioassays with two invertebrate species not only under laboratory

conditions but also in field deployments favour to elucidate different sources of contaminants apart from the sediments permitting a more realistic approach to the original situation of the ecosystem, and the potential ecological risks.

#### **4. Conclusions**

There is evidence that 4 years after the impact of the *Prestige* oil spill the fuel is not producing acute toxic effects on the environment [9] but sub-lethal responses have been detected in the area of Corme-Laxe, related to PAHs, Pb and Hg; no effects of this spill were observed in the study sites located in the AINP although a contamination by metals, specially Zn, Cu and Ni was observed in some sites on Cíes Island. The coastal anthropogenic influence has made evident in both areas due to an input of a mixture of pollutants that should be considered a potential risk. In the case of Corme-Laxe, a possible impact of the mussel rafts was detected.

The use of biomarkers has demonstrated having higher sensitivity than acute toxicity approaches as part of a more complete and integrated study based on a weight-of-evidence approach [9]. The present study has demonstrated the feasibility of incorporating a fourth line of evidence to the classical methodology employed in the Sediment Quality Triad and the suitability of biomarkers as a tool to assess metallic and petrogenic contaminated sediments as well as unknown mixtures of compounds, by carrying out bioassays under field and laboratory conditions which help to distinguish possible sources of pollutants in the environment and provides information about ecological risks.

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# **The application of a weight of evidence approach to compare the quality of coastal sediments affected by acute (*Prestige 2002*) and chronic (Bay of Algeciras) oil spills**

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## **Abstract**

To evaluate sediment quality in different areas affected by oil spills a weight of evidence approach was employed by including a complete set of parameters as part of 4 different lines of evidence: sediment contamination, biological effects and bioaccumulation under laboratory conditions, toxicity in field conditions and benthic alteration. The methodology was applied to sediments from the Bay of Algeciras (S Spain) chronically impacted by different oil spills, and the Galician Coast (NW Spain) acutely impacted by an oil spill (*Prestige 2002*). Results obtained have elucidated the sources and fates of pollutants and the type of risk involved for the ecosystem. It has been demonstrated that the impact associated with chronic event of contamination by oil spills are significantly more dangerous and polluted than those related to acute effects. In the acute events it has been shown that the original pollution is recovered years later whereas the pollution still in those chronic affected environments.

*Keywords: sediment contamination, sediment toxicity, Sediment Quality Triad, bioaccumulation, sublethal, benthic alteration.*

*Capsule: Chronic inputs due to the continuous entrance of contaminants result much more harmful in coastal ecosystems than major but precise environmental impacts*

## **1. Introduction**

Nowadays, human activities in coastal areas involve a high pressure and a source of different contaminants to the natural environment that becomes evident in the decreased quality of coastal sediments. Sediments act as a trap of contaminants and may become sufficiently polluted to disrupt natural biological communities (Adams et al. 1992; Tolun et al. 2001). Substances introduced into the environment may be more or less bioavailable to organisms depending on their chemical form, modifying factors in the environment, the environmental compartment they occupy, and the reactions (behavioural and physiological) of exposed biota (Chapman et al., 2003; Chapman, 2007). The biological effects can be established based on laboratory tests that determine toxic responses, besides, field data on the communities living in the sediments allow to establish whether there is observable pollution-induced degradation effect in the biota (Chapman et al., 1991).

Integrated studies use different lines of evidence (LOEs) which address different questions about the presence of contaminants, their bioavailability and their adverse biological effects (Riba et al., 2004) in a weight of evidence (WOE) framework. In the present study a WOE following 4-LOEs has been applied to compare the sediment quality of two areas of the Spanish Coast affected by oil spills. The Bay of Algeciras (S Spain) has suffered a chronic impact lasting several decades, caused by the input of oil and other contaminants from the various industries located in the area and from accidental spills and deliberate discharges from commercial shipping activities (Morales-Caselles et al., 2007),

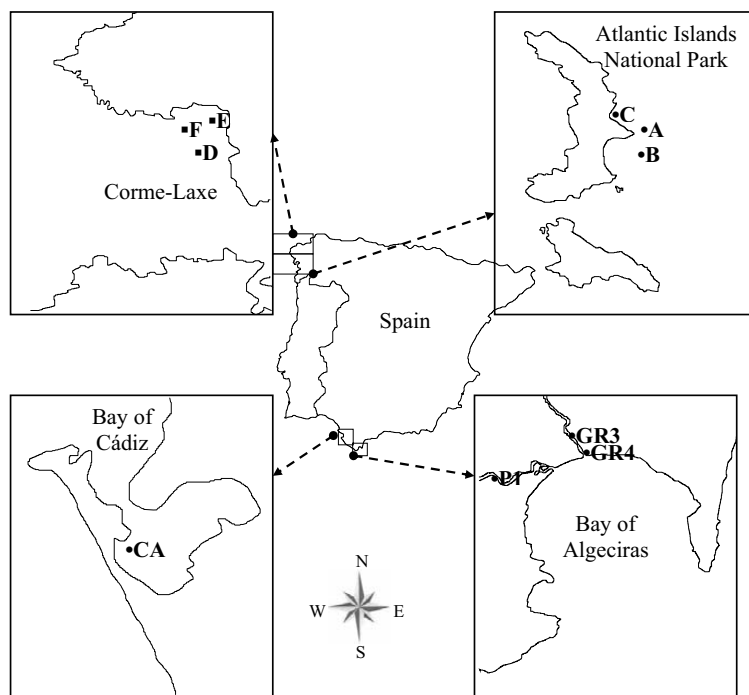
whereas the Galician Coast was impacted by the sinking of the tanker *Prestige* (2002), which spilt about 63,000 tonnes of heavy fuel oil (Mariño-Balsa et al., 2003; Blanco et al., 2006; Fernández et al., 2006). In addition a third area located in the Bay of Cádiz (SW Spain) and widely characterized by different ecotoxicological studies was selected as the reference site (Del Valls et al., 1998, Riba et al., 2004, Martín-Díaz et al., 2005; Morales-Caselles et al., 2007).

The aim of this study are: (a) to determine the feasibility of using the selected parameters as part of 4 LOEs to assess sediments contaminated by different types of oil spills; (b) to establish the environmental degradation in the studied areas; and (c) to elucidate what is more harmful to the environment: acute or chronic impacts associated with oil spills.

## 2. Methodology

### 2.1. Approach

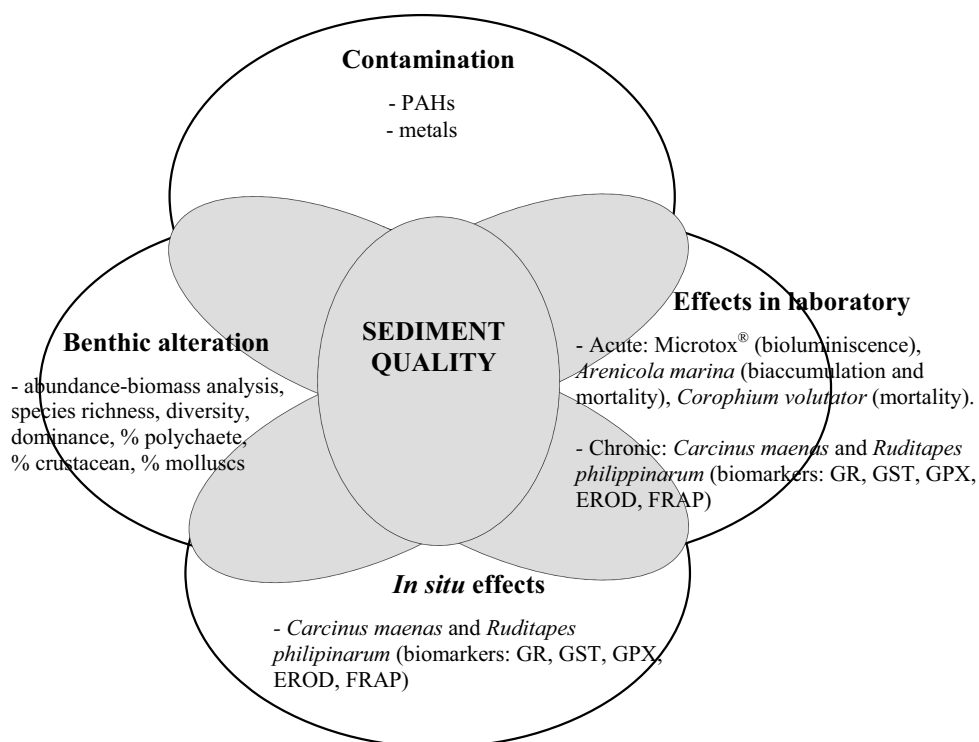
Fig. 1 shows the 6 sediment sampling stations located in the area of Galicia (NW Spain), three stations in the Atlantic Islands National Park (A, B and C) and three stations in the Bay of Corme-Laxe (D, E, F). Both areas were importantly affected by the *Prestige* oil spill and are considered of high ecological importance. In the Gulf of Cádiz (S Spain) three stations were selected in the area of the Bay of Algeciras (GR3', GR4, P1) which is highly industrialized place where it take place a large number of petrochemical activities that comprise several accidental oil spills; besides, a reference site was chosen in a clean area in the Bay of Cádiz (CA) (Riba et al., 2003).



**Figure 1.** Map of the coastal area of Galicia, the Bay of Algeciras and the Bay of Cádiz showing the general areas sampled and locations of the sampling stations. A, B and C are the stations located in the Cies Island in the Atlantic Islands National Park (Galician Coast); D, E and F are the sites from the Bay of Corme-Laxe (Galician Coast); GR3, GR4 and P1 are located in the Bay of Algeciras whereas the reference station CA is placed in the Bay of Cádiz.

## 2.1. The WOE components

A weight-of-evidence approach (WOE) was conducted in the sites selected that includes 4 lines of evidence (LOEs) incorporating the next analysis (Figure 2):



**Figure 2.** Summarized description of the 4 lines of evidence selected in the weigh of evidence approach using the schematic representation modification of the classical triad.

(a) sediment contamination: includes the concentration of total PAHs (acenaphtalene, acenaphtylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i) perylene, benzo(k)fluoranthene, chrysene, dibenzo(a,h) anthracene, fenanthrene,

fluoranthene, fluorene, indene (1,2,3,cd )pyrene, naphthalene, and pyrene) and trace metals (Zn, Pb, Cu, Ni, Co and V). Sediment characterization by organic carbon and percentage of fines is also included in this section (methodologies described in Morales-Caselles et al., 2006);

(b) sediment toxicity under laboratory conditions: including the bacteria assay Microtox® (Morales-Caselles et al., 2007), the amphipod mortality test with *Corophium volutator* (Morales-Caselles et al., 2007) and the polychaeta mortality and bioaccumulation assay (Casado-Martínez et al., in press) with *Arenicola marina*; sublethal assays were also conducted based on biomarkers by using two invertebrate species, the crab *Carcinus maenas* and the clam *Ruditapes philippinarum*, and a suite of biomarkers measured after 28 days of exposure: Ethoxyresorufin O-deethylase (EROD), phase I detoxification enzyme implicated in monooxygenation reactions of dioxins and PAHs; glutathione-S-transferase (GST) phase II detoxification enzyme but also implicated in oxidative stress events; glutathione peroxidase (GPX) and glutathione reductase (GR), antioxidant enzymes (Martín-Díaz et al., 2007); Ferric reducing ability of plasma (FRAP) assay as a measure of antioxidant capacity (Benzie and Strain, 1996); and the vitellogenin variation in crabs (Martín-Díaz, 2004).

(c) Field bioassays were carried out to determine the “in situ” effects. These toxicity tests were performed using field deployments in cages of the crab *Carcinus maenas* and the clam *Ruditapes philippinarum*. The same suite of biomarkers described above and used under laboratory conditions was employed to determine sublethal effects in the organisms exposed during a 28-d period (Martín-Díaz et al., 2007).

(e) ‘in situ alteration’: Benthic alteration was selected and determined by measuring parameters in situ based in taxonomic identifications and

community descriptive statistics (abundance-biomass analysis, species richness, diversity, dominance and proportions of the major taxonomic groups).

## 2.2. Data integration

The integration of the data obtained from the 4-LOEs was performed through a multivariate analysis approach based on linking all the variables obtained which determines the environmental degradation of the studied ecosystems (Riba et al., 2004) and (b) a representation using pie charts by an ANOVA approach and by means of the determination of different factors (Riba et al., 2004; Morales-Caselles, accepted). The multivariate analysis was performed using principal components analysis (PCA) in order to derive a reduced number of new variables (factors) as linear combinations of the original variables. This provides a description of the structure of the data with the minimum loss of information (Riba et al., 2003). Pie charts were obtained by conducting an ANOVA and Tukey tests which identified significant differences ( $p < 0.05$ ;  $p < 0.01$ ) in sensitivity among stations and the reference station for each of the factor scores obtained from the PCA (Morales-Caselles et al., accepted).

## 3. Results

Table 1 shows the summarized results of the different parameters analyzed in the study. In general, no organic contamination was observed in the reference site whereas the highest levels of PAHs were detected in stations from the Bay of Algeciras. The concentration of metals in sediments varies among the sites and the organic carbon and fines contents are higher in sediments collected on Algeciras. It was not observed a general pattern in biological parameters between the sampling sites except for site GR3 which

presented the highest mortality in the acute bioassays and also a remarkable benthic alteration. To elucidate the matrix of data the PCA was performed to link the variables included in the 4 LOEs (contamination, effects under laboratory conditions, *in situ* effects and benthic alteration) applied to determine the sediment quality of the two areas affected by oil spills we have obtained five new factors that account for all the variables and have a different influence for each sampling site (table 2). These factors explain an 82.7 % of the variance in the original data set, and negative loading are considered as important as positive values. The predominant factor, Factor #1, accounts for a 34.5 % of the variance and shows the relationship between the concentration of Pb, Ni and PAHs in sediment, the percentage of organic carbon and fines, the lethal toxicity determined by the amphipod bioassay, the bioaccumulation of PAHs in *A. marina*, the induction of GPX (crabs), EROD (crabs and clams), vitellogenin variation (crab) and FRAP (clam) activity under laboratory conditions and the induction of EROD activity (clams) after field exposures; besides all the variables related with benthic alteration (abundance, species richness, diversity, dominance and proportions of molluscs, polychaete and crustacean) are gathered in Factor #1. Taking this into account this factor represents environmental degradation by PAHs, Pb and Ni.

Factor #2 (18.0 %) combines, with negative loading, the presence of Hg in the sediments with a set of biomarkers (GPX and GST activity in crab and GR induction in clams under laboratory and field conditions, GPX, GR and GST in crabs after field exposures, GR and FRAP activity in clams under laboratory conditions and inverse relation with GST in clam in laboratory studies and FRAP in crabs under field deployments) and the variables of benthic alteration described by species richness and percentage of crustacean. This factor can be explained as pollution produced by the presence of Hg in the sediments.

**Table 1.** Summarized results of physicochemical analysis ( $\text{mgKg}^{-1}$  for metals,  $\mu\text{gKg}^{-1}$  for PAHs, percentage of organic carbon –o.c.- and fines in sediment) the acute toxicity tests (Corophium and Arenicola: % mortality; Microtox: IC50; bioaccumulation of PAHs:  $\mu\text{gKg}^{-1}$ ), biomarker responses under field and laboratory conditions (glutathione peroxidase activity GPX:  $\text{nmol/min/mg prot}$ , glutathione transferase GST activity  $\text{nmol/min/mg prot}$ , glutathione reductase GR activity  $\text{nmol/min/mg prot}$ , ferric reducing ability of plasma FRAP activity  $\mu\text{M/mg/min}$ , EROD activity  $\text{pmol/mg/min}$  and vitellogenin variation  $\text{ng } 100\text{mL}^{-1}$ ) and the alteration parameters for sediments from the Galician Coast -AINP (A, B, C) and Corme-Laxe (D, E, F)- and the Gulf of Cádiz –Bay of Algeciras (GR3, GR4, P1) and the Bay of Cádiz –CA-. n.d.: not detected; n.a.: not available.

		A	B	C	D	E	F	GR3	GR4	P1	CA
Physicochemical analyses	Zn	377	91	164	25	19.9	271	138	35.3	56.7	21.3
	Pb	1.5	0.9	0.85	3.7	7.3	5.9	21.6	6.21	12.3	2.28
	Cu	5.2	1.4	1.4	0.7	0.43	4.2	5.01	3.67	75.2	6.98
	Ni	13.3	2.4	4.5	1.7	1.5	5.7	74.7	13.1	13.3	0.06
	Hg	0.7	0.8	0.6	2	2.1	3.4	1.04	0.25	0.65	n.d.
	PAH	108	67	n.d.	38	52	323	2961	802	641	n.d.
	O.C.	0.28	0.26	0.30	0.31	0.37	0.65	2.15	3.19	3.86	1.07
	Fines	4.32	2.81	2.76	3.79	5.50	5.95	69.35	59.33	35.44	2.5
Toxicity tests	Corophium	23	20	17	10	17	20	100	75	20	0
	Arenicola	28	28	22	39	17	17	30	17	46	0
	Microtox	5631	9422	1801	3977	21041	4398	235	249	1642	6013
	Bioaccumulation PAH	2927	2573	2666	2616	3912	3285	5158.9	4809.1	4097.0	2421.0
Biomarkers (laboratory)	GPX-crab-lab	11.6	9.7	8.2	19.3	19.5	15.9	18.8	9.1	12.4	6.3
	GPX-clam-lab	2.1	2.9	4.5	6.1	3.1	4.2	3.8	2.7	2.5	5.1
	GR-crab-lab	1.1	0.7	0.9	0.9	0.6	1.5	1.1	0.5	1.0	0.9
	GR-clam-lab	2.1	1.6	2.3	3.4	11.7	4.0	2.8	1.4	1.5	2.7
	GST-crab-lab	140	218	407	430	684	1071	294.6	203.2	377.7	611.2
	GST-clam-lab	1293	839	1624	1199	910	848	901.1	1634.5	1117.7	1542.1
	EROD-crab-lab	0.1	0.1	0.1	0.0	0.1	0.1	0.3	0.3	0.0	0.0
	EROD-clam-lab	0.3	0.3	0.4	0.4	0.4	0.2	1.2	0.7	0.8	0.1
	FRAP-crab-lab	3.9	2.1	2.6	2.9	2.9	1.6	2.1	n.a.	6.1	1.7
	FRAP-clam-lab	10.6	7.8	4.0	13.7	12.1	6.4	15.4	3.6	8.7	9.5
	VIT-crab-lab	0.0936	0.0380	0.0879	0.1130	0.0889	0.1440	0.3074	0.2332	0.4301	0.0755
Biomarkers (field)	GPX-crab-field	17.8	23.1	15.9	41.4	193.1	125.7	4.8	6.5	4.0	6.3
	GPX-clam-field	10.5	3.6	4.0	25.5	3.2	7.0	2.9	2.8	3.6	5.1
	GR-crab-field	0.7	1.4	1.4	9.9	9.5	23.4	0.8	1.5	0.5	0.9
	GR-clam-field	2.9	1.3	3.8	9.7	14.7	8.0	1.9	1.1	0.8	2.7
	GST-crab-field	1098	1564	690	1489	7523	6073	443.4	1352.7	592.7	611.2
	GST-clam-field	2061	372	1199	3366	131	1558	997.8	1031.6	876.5	1542.1
	EROD-crab-field	0.1	3.0	0.0	8.5	0.4	0.5	0.0	0.0	0.0	0.0
	EROD-clam-field	0.2	0.1	0.1	0.6	0.1	0.1	0.3	0.3	0.3	0.1
	FRAP-crab-field	2.7	n.a.	n.a.	2.4	n.a.	n.a.	1.9	3.5	4.2	1.7
	FRAP-clam-field	10.4	3.1	2.6	23.6	2.0	6.6	2.4	7.7	4.1	9.5
	VIT-crab-field	0.0049	0.0099	0.0879	0.0047	0.0426	0.0098	0.0189	0.1652	0.0303	0.0721
Benthic alterations	species N°	28.5	33.9	42.4	28.6	32.1	48.2	0.67	4.67	4.67	14
	specific richness	5.1	5	4.3	3	3	2.9	0	1.21	1.25	2.57
	Diversity	15.3	28.4	39.1	30	40.1	15.4	0	1.29	1.24	1.64
	Dominance	0.50	0.10	0.06	0.15	0.19	0.20	0	0.72	0.68	0.66
	% Mollusca	15.3	28.4	39.1	30.0	40.1	15.4	0.0	34.4	25.4	78.5
	% Polychaete	20.0	21.5	21.7	20.0	22.2	23.1	100.0	45.3	64.4	12.7
	% Crustacea	37.0	41.0	39.1	50.0	51.4	61.5	0.0	20.3	10.2	8.8

**Table 2.** Sorted rotated factor loadings for the five principal factors obtained after applying the principal components analysis to the original data set of 41 parameters included in the weight of evidence approach.

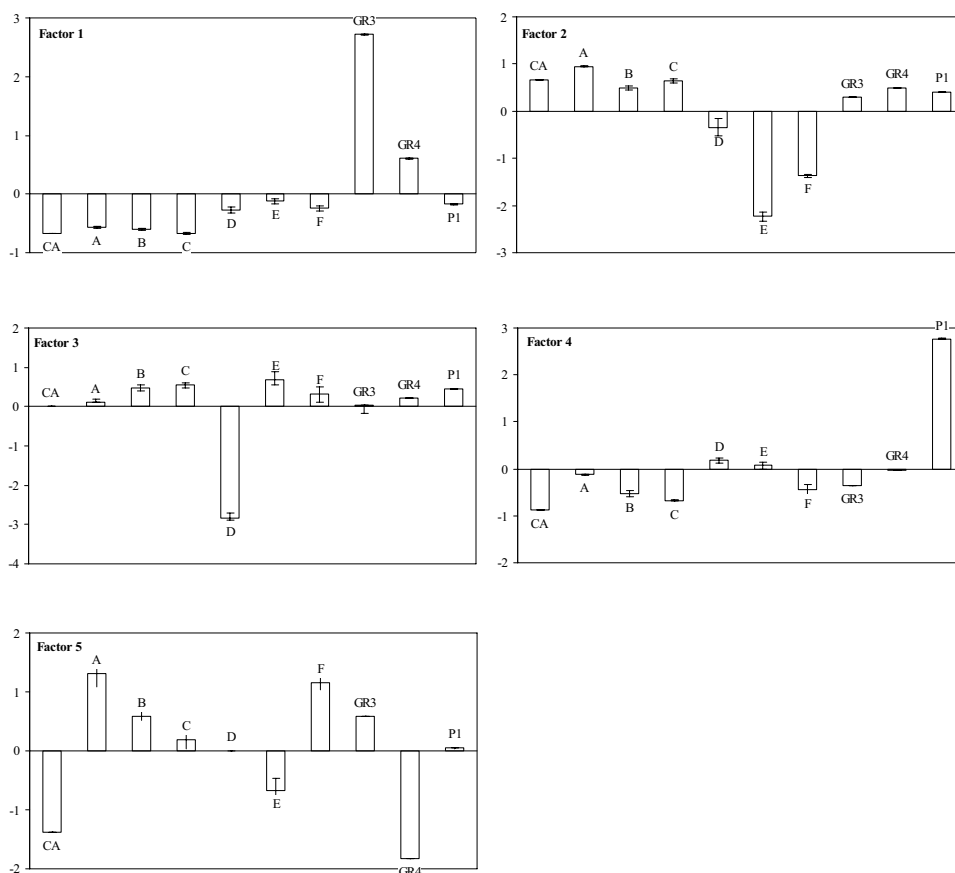
	<b>Factor 1</b>	<b>Factor 2</b>	<b>Factor 3</b>	<b>Factor 4</b>	<b>Factor 5</b>
	34.5	18.0	14.0	9.0	7.1
Zn	—	—	—	—	0.78
Pb	0.90	—	—	0.35	—
Cu	—	—	—	0.93	—
Ni	0.95	—	—	—	—
Hg	—	-0.81	—	—	0.43
PAH	0.98	—	—	—	—
O.C.	0.45	—	—	0.67	-0.38
Fines	0.86	—	—	—	—
Corophium	0.92	—	—	—	—
Arenicola	—	—	-0.35	0.70	0.43
Microtox	—	—	—	-0.30	-0.47
Bioaccumulation PAHs	0.83	—	—	—	—
GPX-crab-lab	0.47	-0.46	0.68	—	—
GPX-clam-lab	—	—	-0.57	-0.31	—
GR-crab-lab	—	—	—	—	0.64
GR-clam-lab	—	-0.87	—	—	—
GST-crab-lab	—	-0.72	—	—	—
GST-clam-lab	—	0.50	—	—	-0.49
EROD-crab-lab	0.85	—	—	—	—
EROD-clam-lab	0.88	—	—	0.38	—
FRAP-crab-lab	—	—	—	0.77	0.39
FRAP-clam-lab	0.45	—	-0.45	—	—
VTG-crab-lab	0.55	—	—	0.77	—
GPX-crab-field	—	-0.96	—	—	—
GPX-clam-field	—	—	-0.94	—	—
GR-crab-field	—	-0.76	—	—	—
GR-clam-field	—	-0.88	—	—	—
GST-crab-field	—	-0.94	—	—	—
GST-clam-field	—	—	-0.73	—	—
EROD-crab-field	—	—	-0.89	—	—
EROD-clam-field	0.30	—	-0.86	0.34	—
FRAP-crab-field	—	0.47	—	0.65	—
FRAP-clam-field	—	—	-0.93	—	—
VTG-crab-field	—	—	—	—	-0.80
species N°	0.96	—	—	—	—
specific richness	0.52	0.42	—	0.36	-0.54
Diversity	0.93	—	—	—	—
Dominance	0.93	—	—	—	—
% Mollusc	0.55	—	—	—	0.70
% Polychaeta	0.89	—	—	0.38	—
% Crustacea	0.53	0.60	—	—	-0.35

The third factor, Factor #3, accounts for a 14.0 % of the variance and links, with negative loading, the toxicity detected by the *Arenicola* assay, with the induction of some biomarkers mainly under field conditions (GPX and FRAP in clam in field and laboratory exposures; GST induction in clam and EROD activity in clam and crabs under field conditions; opposite link with GPX activity in crabs from the laboratory experiments). This factor is related to an unknown stressor which is producing a general stress to the exposed organisms but not a benthic alteration neither a pollution nor degradation in the benthic environment.

Factor #4 (9.0 %) is a combination of the concentration of Pb and Cu in the sediments, with the percentage of organic carbon, toxicity in the *Arenicola* toxicity test, *in situ* alteration of the polychaete population and the variation of some biomarkers (EROD in clams and FRAP in crabs under laboratory and field conditions, Vitellogenin variation in crabs and GPX activity in clams under laboratory conditions). In general, Factor #4 can be related to a contamination by Cu and Pb that can be considered a potential risk to the environment but not associated with pollution.

Factor #5 represents a 7.1 % of the variance and groups the metals Zn and Hg bound to sediment with toxic responses in the *Arenicola* toxicity test, the antioxidant activity determined by the induction of GR and FRAP in crabs in laboratory exposures and the alteration of the mollusc population. Other variables present opposite behaviour such as the GST in clams and vitellogenin variation in crabs, specific richness and the percentage of crustacean. In this sense, this factor could be explained as a contamination by Zn and Hg which is producing some stress in the environment but not pollution.

In order to establish the meaning of each factor in the area of study, the factor scores have been represented for every single station (Figure 3). Factor #1

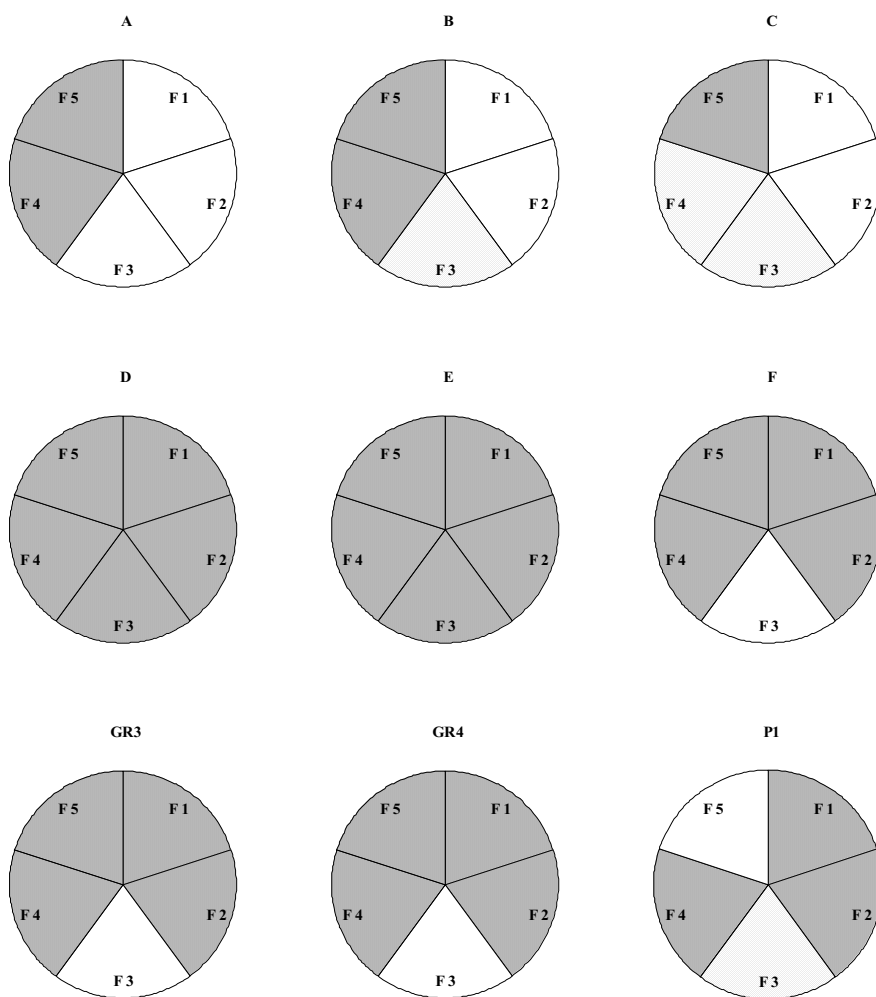


**Figure 3.** Estimated factor scores for the three factors in each of the 10 cases. The factor scores quantify the prevalence of each factor for every station.

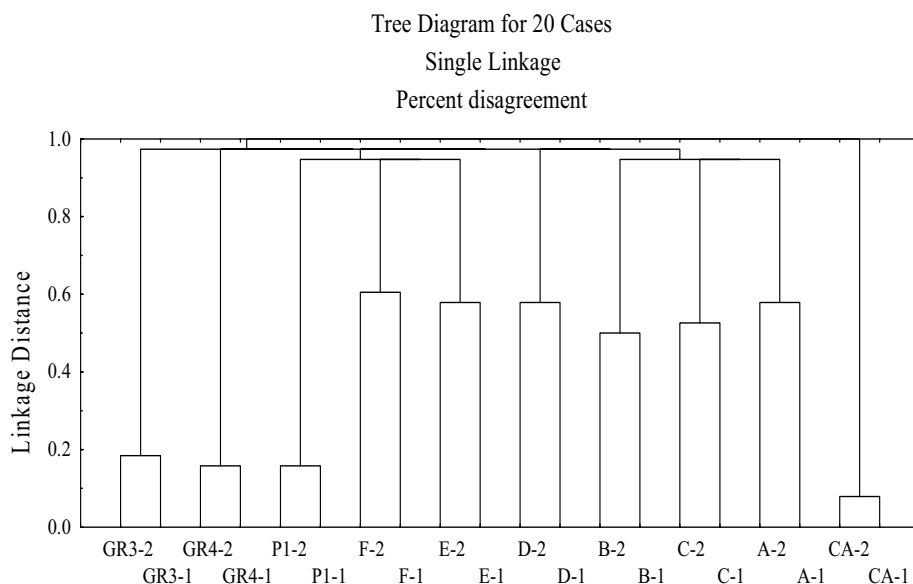
related to the environmental degradation by PAHs, Pb and Ni presents a positive loading mainly in the station GR3 (2.7) followed by the site GR4 (0.6) from the Bay of Algeiras. The second factor which explains with negative loading the stress produced by the presence of Hg in the sediments shows prevalence in the study sites located in the Bay of Corme-Laxe E (-2.2) > F (-1.4) > D (-0.3). The unknown stressor described by Factor #3 with negative loading has only prevalence in the station D (-2.8) from Corme-Laxe. In the case of Factor #4 related to a contamination by Cu that which could be considered a

potential risk, the affected are slightly stations D (0.2) and E (0.1) located in the area of Corme-Laxe and mainly station P1 (2.8) located in the Bay of Algeciras. Finally, the prevalence of Factor #5, which explains the stress caused by the contamination by Zn and Hg, is detected in the stations located in the AINP A (1.3), B (0.6) and C (0.2), the site F (1.1) in Corme-Laxe and GR3 (0.6) in the Bay of Algeciras.

Figure 4 shows the significant differences between the stations and the reference site for each of the five studied factors. Significant differences ( $p < 0.01$ ) from the reference (CA) were observed for Factor #1 and #2 in the stations from Corme Laxe (D, E, F) and the Bay of Algeciras (GR3, GR4 and P1). Factor #3 was significantly different for B, C ( $p < 0.05$ ) in Cies, D ( $p < 0.01$ ), E ( $p < 0.01$ ) in Corme-Laxe and P1 ( $p < 0.05$ ) in Algeciras. On the other hand Factors #4 and #5 resulted to be significantly different ( $p < 0.01$  except for F#4 in C which presented  $p < 0.05$ ) to the reference for all the studied stations except for Factor #5 in P1 which did not present these differences. According to the Cluster analysis (Figure 5) the stations were grouped in a way similarly to their real location in field.



**Figure 4.** Pie charts which represent the significant differences of the factors score in every study site related to the reference site CA (dotted:  $p < 0.01$ ; slightly dotted:  $p < 0.05$ ; not dotted: no significant differences,  $p > 0.05$ ).



**Figure 5.** Tree diagram classification of the 10 stations (in duplicate) based in Cluster analysis (CA: reference station; A, B and C: AINP; D, E and F: Corme-Laxe; GR3, GR4 and P1: Bay of Algeciras).

## 4. Discussion

In the present study the integration of 4 LOEs as part of a WOE approach to assess oil contaminated sediments is proposed. The different lines employed include a set of 41 variables related to contamination, toxicity and bioaccumulation under laboratory conditions, sediment toxicity measure under field conditions and benthic alteration analyzing the macrobenthic structure parameters. The use of sublethal bioassays validated both in laboratory and field exposures by using biomarkers contributes to a better understanding of the toxic processes of the contaminants and supplies the lack of information often shown by acute toxicity tests performed alone. The application of this methodology to sediments affected by oil spills in different manners has allowed determining the environmental quality of the impacted areas as well as differentiating the most probable causes of the environmental degradation.

The Multivariate analyses have demonstrated the suitable use of the site CA as reference station. Results have shown that the Galician Coast which was affected by the oil spill from the tanker *Prestige* in 2002 does not present an environmental degradation due to hydrocarbons when comparing with the Bay of Algeciras four years after the spill; however significant differences ( $P < 0.01$ ) were detected with the reference station regarding to sediments pollution due to fuel oil in the Bay of Corme-Laxe (Morales-Caselles et al submitted). On the other hand the study sites located in the Cies Island in the AINP present absence of pollution due to fuel oil stem from the tanker *Prestige* although an environmental risk caused by a metallic contamination of Cu, Zn and Hg is present in the area. Previous studies have demonstrate sources of metals coming from anthropogenic sources located in the area closed to the AINP (Carballeira et al., 1997; Pérez-López et al., 2003). The Bay of Corme-Laxe also presents environmental stress due to Cu, Zn and Hg coming from anthropogenic sources which could include different kind of spills coming from land and the maritime traffic. Even though no signals of alteration of the benthic community have then detected in the area, a non quantified stressor has been determined as potentially toxic. The stress was mainly detected under field exposures what suggests that the stressor could came from the water; a possible cause could be related to the presence of industrial cultured of caged mussels specially closed to station D which imply a source of organic matter to the water column providing stress to the organisms exposed. On the other hand, the results observed in the study sites from the Bay of Algeciras have shown an important environmental degradation in the Guadarranque River due to a chronic contamination by a mixture of contaminants that include mainly Pb, Ni and PAHs, all of them representative of hydrocarbon contamination in the ecosystem and previously reported by other studies (CSIC, 2003; CSIC, 2005). Acute and sublethal toxicological responses besides an important alteration on the biota were associated with this kind of pollution. The presence of petrochemical industries, the high maritime traffic and the

bunkering activities are the main factors which involve a threat to the marine ecosystem of the Bay of Algeciras in addition to the human risk represented by the collection of goods for consume in the zone. Other contaminants such as Cu, Zn and Hg are producing stress to the biota in stations P1 and GR3; on the contrary, the site GR4, which is located in the mouth of the river, does not present this environmental pressure by metals what suggests that the pollution by these contaminants might come from direct spills to the rivers from the industries located in the area. Despite the tide regime of the area which implies an important water renewal, the degradation of the ecosystem in the mouth of the River Palmones and Guadarranque is a fact. Taking this into account, it is possible to have other types of contaminants in the area which may be contributing to the environmental impact and not measured in this study (Antón, 2007). The cluster analysis have confirmed the disparity of the stations from the Bay of Algeciras > Corme-Laxe > AINP > reference station.

Regarding to the obtained results, the recovery of the Galician coast affected by the *Prestige* oil spill is significantly notable (Morales-Caselles et al., accepted; Morales-Caselles et al., submitted) although other sources of contaminants should be taken into consideration due to the potential risk that involve. On the other hand, the chronic pollution in the Bay of Algeciras which is not only composed by hydrocarbons spills but with the existence of a complex mixture of contaminants inputs, is producing a considerable additionally damage to the ecosystem. In this sense, chronic inputs due to the continuous entrance of contaminants result much more harmful in coastal ecosystems than major but precise environmental impacts, as confirmed in previous studies (Riba et al., 2004).

## 5. Conclusions

In the present study authors have successfully integrated 4 LOEs in a WOE approach to assess sediments affected by oil spills and different sources of

contaminants. The use of physicochemical characterization, biological responses under laboratory and field conditions and *in situ* alteration of the biota as part of a WOE approach is considered a suitable tool to carry out sediment quality studies. This methodology based on the evaluation of a complete set of parameters under an integrated framework goes further than the classical studies by studying the real status of the environment and including early warning signals of risk. The combination of field and laboratory analysis supposes an added value to the assessment whereas the complete methodology employed has elucidated about the contaminants sources and fates, in addition to their implication as an environmental risk.

The existence of a wide group of sources in the Bay of Algeciras including urban and industrial activities in addition to the maritime traffic which involve accidental spills makes difficult to elucidate the main cause of the environmental health decrease. Results obtained indicate that the high environmental degradation present in the Bay of Algeciras is mainly due to continuous oil spills. On the other hand, four years after the *Prestige* oil spill, a general recovery of the sediments affected in Atlantic Islands National Park (AINP) and an improvement in the environmental quality in the Bay of Corme-Laxe was observed. Other inputs of contaminants not related with oil spills were also detected in these areas; at the moment these sources of stress are not producing damage to the biota although they constitute an environmental risk that should not be ignored.

To sum up, the environment capacity of recoverment after a major oil spill episode such as the *Prestige* has been demonstrated whereas littoral sediments affected by low or moderated but continuous oil spills have resulted to be more degraded. This conclusion should lead to the reflection on our perception and major concerns of the environmental pollution.

## 6. Acknowledgements

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## Capítulo 6.

### Conclusiones

1) Se han determinado las concentraciones de metales y contaminantes orgánicos en diferentes sedimentos de la costa Gallega localizados en el Parque Nacional de las Islas Atlánticas (islas de Ons y Cies) y en la Bahía de Corme-Laxe tras el vertido del petrolero *Prestige* y se han comparado con niveles de estos contaminantes en zonas del Golfo de Cádiz. La presencia de niveles elevados de PAHs en sedimentos junto con los niveles moderados de metales en algunas de las zonas estudiadas en Galicia, ponen de evidencia la influencia del vertido de petróleo en la zona. Los niveles de contaminantes en la zona de la Bahía de Algeciras confirman su carácter de zona contaminada en varios de los puntos estudiados, tanto por metales como por contaminantes orgánicos. La estación seleccionada en la Bahía de Cádiz ha resultado ser adecuada como referencia dados sus bajos niveles de contaminación metálica y la ausencia de PAHs y PCBs.

2) Se ha identificado y cuantificado el efecto adverso de los contaminantes presentes en el fuel procedente del petrolero *Prestige*, demostrando que la

especie *Ampelisca brevicornis* es adecuada para realizar ensayos de toxicidad a la hora de determinar la toxicidad aguda en sedimentos afectados por contaminación de tipo orgánico y metálico.

3) Se ha demostrado la ventaja de utilizar el poliqueto *Arenicola marina* en la caracterización de zonas afectadas por vertidos de petróleo, y que a pesar de ser una especie menos sensible que otras *A. marina* puede estar presente en áreas polucionadas lo que permite realizar estudios de bioacumulación. Los PAH procedentes del fuel del *Prestige* que más se bioacumularon fueron fluoranteno, pireno benzo(fluoranteno y benzo(k)fluoranteno mientras que fenantreno y antraceno se acumularon inicialmente y luego fueron probablemente metabolizados.

4) A partir de los ensayos de toxicidad se ha detectado una respuesta aguda moderada-baja debido principalmente a los PAHs originados tras el vertido en los sedimentos del Parque Nacional de las islas Atlánticas; por otra parte, la Bahía de Algeciras presentó elevada toxicidad aguda mientras que no se detectaron efectos adversos en los organismos expuestos a sedimentos procedentes de la estación de referencia localizada en la Bahía de Cádiz.

5) Los resultados obtenidos demuestran que existe una disminución de la toxicidad aguda en los sedimentos afectados por el vertido del petrolero *Prestige* en la costa de Galicia; los sedimentos de Corme-Laxe y el Parque Nacional de las Islas Atlánticas no presentaron toxicidad aguda cuatro años después del vertido aunque la presencia de algunos metales y PAHs en los sedimentos es considerada como un riesgo potencial para la calidad de los mismos. La bioacumulación de PAHs en poliquetos expuestos a sedimentos de Corme-Laxe indica la posibilidad de que se den efectos subletales en los organismos.

6) Con el fin de realizar un estudio más completo de la toxicidad de los sedimentos afectados por vertidos de petróleo se han diseñado una serie de bioensayos con invertebrados y vertebrados marinos que mediante exposiciones subletales y medidas de diferentes biomarcadores han permitido diferenciar “zonas grises” en la clasificación de toxicidad. Los resultados obtenidos muestran una clara diferenciación entre los sedimentos recogidos en el Parque Nacional de las Islas Atlánticas, la Bahía de Corme-Laxe. En este sentido, estos ensayos y medidas utilizadas en los mismos han demostrado ser útiles para la clasificación de la calidad de los sedimentos estudiados.

7) Se han determinado los efectos adversos de los contaminantes en los sedimentos exponiendo durante 60 días juveniles del pez *Sparus aurata* a éstos mediante la aplicación de un bioensayo de toxicidad crónica y analizando diferentes biomarcadores de exposición a metales (Metalotioneinas) y a contaminantes orgánicos (Actividad EROD) y biomarcadores de efecto (histopatología en dos tejidos, branquias e hígado). Los resultados después de los 60 días muestran una correlación con los datos de toxicidad aguda y confirman la presencia de elevadas concentraciones de PAHs en sedimentos como la causa de los efectos analizados tras el vertido en estaciones del Parque Nacional de las Islas Atlánticas

8) Se ha caracterizado la relación de los biomarcadores de exposición a contaminantes orgánicos (Actividad EROD) y la de los biomarcadores de efecto (histopatología) a lo largo del tiempo mediante la aplicación de un modelo cinético. La inducción de la actividad EROD a lo largo del tiempo es mayor en las estaciones con mayor concentración de PAHs. El modelo predice la relación entre la inducción de la actividad EROD como el primer sistema de defensa enzimático frente a la presencia de éstos contaminantes y además determina su eficiencia frente a la aparición del daño histopatológico provocando su

inducción un retraso en la aparición del daño. La aparición de estos daños histológicos es más severa una vez que la inducción de actividad EROD se estabiliza o disminuye en las tres estaciones del Parque de las Islas Atlánticas utilizadas en este estudio.

9) Se ha demostrado como los biomarcadores medidos en la almeja *Ruditapes philippinarum* y el cangrejo *Carcinus maenas* se activan en función del tipo y el nivel de contaminación en los sedimentos, permitiendo establecer diferencias en función del origen y fuente de los contaminantes.

10) Tras varios años después del vertido del petrolero *Prestige* los sedimentos del Parque Nacional de las Islas Atlánticas presentaron los niveles más bajos de respuesta subletal, mostrando una recuperación de la zona tras el vertido; sin embargo, la presencia de algunos metales ligados al sedimento podrían acarrear cierto estrés ambiental en el parque. Los organismos expuestos en jaulas ancladas en la zona de Corme-Laxe han mostrado niveles elevados de este tipo de estrés, lo cual no se observó en los ensayos de laboratorio lo que revela el impacto de otras fuentes de contaminación en la zona. En el caso de la Bahía de Algeciras la inducción de los biomarcadores fue significativa bajo condiciones de laboratorio mientras que en las exposiciones en campo se observó una disminución de los mismos, posiblemente relacionada con la influencia mareal o el efecto de “lavado” de la contaminación, que disminuiría la biodisponibilidad de los contaminantes. En el caso de la vitelogenina medida en cangrejos, para todas las estaciones ésta mostró mayor respuesta bajo condiciones de laboratorio, mientras que las exposiciones en jaulas resultaron menos sensibles a la toxicidad de los sedimentos. Los resultados obtenidos ponen de manifiesto la importancia de realizar exposiciones en campo complementarias a aquellos ensayos de laboratorio.

11) Los resultados de biomarcadores obtenidos en los diferentes organismos expuestos a sedimento de la Bahía de Cádiz, confirman su elección como referencia en estudios de toxicidad, dada la ausencia de respuestas biológicas adversas. Y confirman esta zona como de referencia para estudios que impliquen evaluaciones de toxicidad mediante ensayos crónicos y utilizando medidas subletales como los biomarcadores.

12) Los biomarcadores analizados en cangrejos y almejas se indujeron de manera significativa durante la primera semana de exposición. Se han observado relaciones entre biomarcadores de la fase I y II de detoxificación lo que sugiere la funcionalidad del este sistema en ambas especies de invertebrados marinos. El estudio de biomarcadores a lo largo del tiempo ha ayudado a identificar tanto las fuentes como la manera de actuar y detoxificar los contaminantes, así como de identificar respuestas biológicas frente a contaminantes que no han sido analizados.

13) Se ha demostrado como varía la biología del poliqueto *Arenicola marina* expuesto a diferentes niveles de contaminación. Se han establecido correlaciones entre contaminantes orgánicos, metales y respuestas biológicas, incluyendo actividad antioxidante, inmune y químico-sensorial siendo el biomarcador más destacable el daño de ADN medido por el ensayo Cometa. Por vez primera se ha seleccionado y aplicado una batería de biomarcadores con el poliqueto *A. marina* y los resultados obtenidos muestran nuevas herramientas para su aplicación en estudios medioambientales.

14) Se ha demostrado la recuperación de la fauna bentónica años después del vertido del *Prestige* en la zona de Galicia. Se ha demostrado una importante alteración biológica en las estaciones localizadas en la bahía de Algeciras y se han relacionado con la concentración de PAHs en el sedimento. A su vez, las

comunidades bentónicas de Corme-Laxe y Algeciras, pueden verse afectadas por la presencia de metales en los sedimentos. La estructura de la comunidad bentónica en la zona de Cádiz se estableció como normal no asociándose con alteración significativa, al menos en la estación elegida como referencia.

15) Se ha llevado a cabo una nueva mejora en la metodología integrada de evaluación de la calidad de los sedimentos dentro del marco del “Weight of Evidence approach” que ha permitido obtener resultados más objetivos. Se ha demostrado como tras el vertido los PAHs fueron el principal contaminante de la costa gallega. Se ha identificado la existencia de fuentes de metales en el Parque Nacional de las Islas Atlánticas y de la bahía de Corme-Laxe que aparentemente no están produciendo efectos biológicos de tipo agudo. La polución ha disminuido en los últimos años en ambas zonas de Galicia, aunque aún existe cierto estrés ambiental principalmente en las zonas estudiadas de la bahía de Corme-Laxe.

16) Se han demostrado las ventajas de incorporar los biomarcadores como línea de evidencia dentro de un estudio integrado, éstos han mostrado una mayor sensibilidad en los resultados a la hora de cuantificar la polución e identificar la misma; el uso de los biomarcadores obtenidos en exposiciones de campo y laboratorio dentro del “Weight of Evidence approach” ha ayudado a relacionar las fuentes de contaminación y los efectos incluso cuando el contaminante no ha sido analizado. A pesar que varios años después del vertido no se han detectado efectos agudos significativos en el área de Corme-Laxe, se han observado respuestas subletales relacionadas con las concentraciones de contaminantes como los PAHs, y los metales Pb y Hg. La presencia de ciertos metales como Zn, Cu y Ni en las islas Cíes podrían suponer un riesgo aunque por el momento no se han detectado efectos biológicos en la zona asociados con estos contaminantes. Tras los resultados obtenidos en la Bahía de Corme-Laxe,

se sospecha que la presencia de las bateas de cultivo de mariscos pueda suponer una fuente de estrés importante en la zona.

17) La existencia de distintas fuentes de contaminación da lugar a la presencia de una mezcla compleja de contaminantes en la Bahía de Algeciras, incluyendo vertidos industriales, urbanos y derivados del tráfico marítimo y de las actividades de *bunkering*. Todo ello se refleja en una alta degradación ambiental debida a la entrada continuada de estos vertidos. Por otro lado, cuatro años después del vertido del petrolero *Prestige* se observa una recuperación generalizada de los sedimentos del Parque Nacional de las islas Atlánticas y una mejora en la calidad de la Bahía de Corme-Laxe. El método integrado ha demostrado la recuperación del sistema afectado en la costa de Galicia, la polución en la zona de la Bahía de Algeciras y la condición de zona de referencia en la estación elegida en la Bahía de Cádiz.

18) Se ha demostrado la capacidad ambiental de recuperación tras un gran vertido de petróleo como el ocurrido en Galicia en 2002 mientras que sedimentos litorales que se ven afectados por moderadas dosis de vertidos durante un largo periodo de tiempo y que en principio no desatan tanta alarma social pueden resultar notablemente más degradados como es el caso de la Bahía de Algeciras.