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Efecto de los impactos antropogénicos en las relaciones entre especies de ecosistemas marinos

Effect of anthropogenic impacts on the genetic relationships among marine species

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“We should try to leave the world a better place than when we entered it. As individuals, we can make a difference, whether it is to probe the secrets of Nature, to clean up the environment and work for peace and social justice, or to nurture the inquisitive, vibrant spirit of the young by being a mentor and guide.”

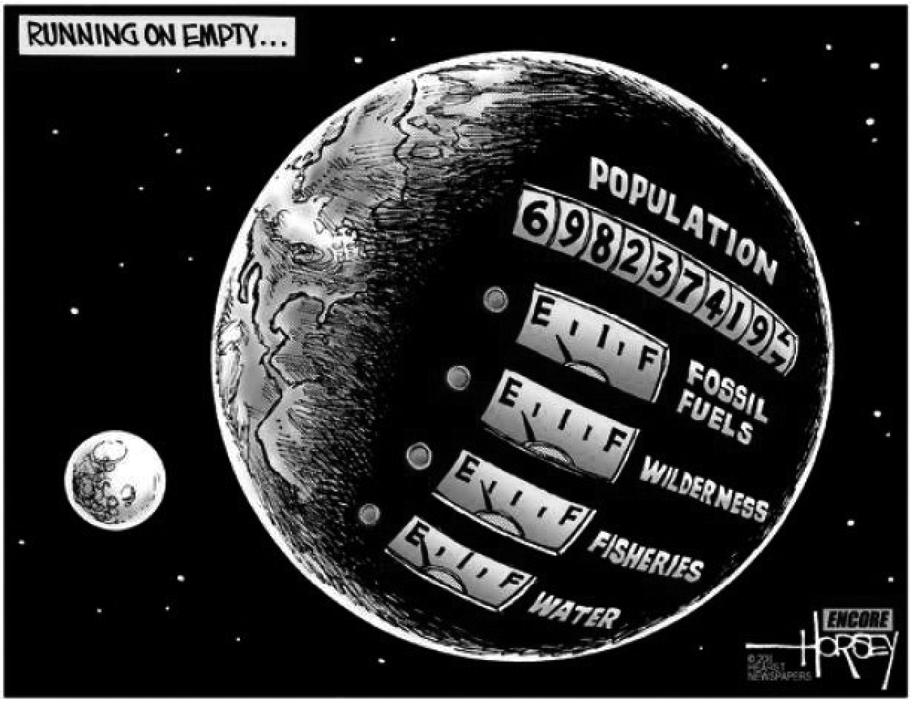
- 加來 道雄 (Kaku Michio) -

“There can’t be any large-scale revolution until there’s a personal revolution, on an individual level. It’s got to happen inside first.”

- Jim Morison -

“Desde el principio de los tiempos, los ríos han sido las arterias que transportaban vida.
Las montañas y la tierra fueron mi piel. Los bosques y la selva, mi pelaje.
Todo estaba en permanente armonía, hasta que apareció el ser más cruel y caprichoso que jamás conoció este planeta: el hombre. Una forma de vida supuestamente inteligente, pero desperdiciada por la codicia, la violencia y la incultura.
Vosotros utilizáis el mar para alimentaros y para viajar por él, pero jamás lo cuidáis. Y el mar, como ser vivo que es, necesita amor y atenciones, al igual que el rio o el árbol.
Yo soy Gaia, la madre naturaleza, y todo el mal que me hagas, te lo devolveré.”

Extracto de Gaia
- Jesús María Hernández Gil -



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Cambiando de tema, una de las partes más tediosas de los trabajos de investigación es la recopilación de muestras. Toda persona que ha realizado esta tarea conoce su complejidad y la angustia que supone necesitar una muestra en concreto y no poder conseguirla. La recolección de muestras es una de las partes más importantes, ya que sin ellas no se podría seguir adelante, y sin embargo luego quedan reducidas a una mera tabla o a una enumeración de materiales. Por ello, aquí quiero agradecer la colaboración de todas las personas que se han dejado convencer y han contribuido en la obtención de las mismas y/o realización de los muestreos, en especial a los integrantes de la Cofradía de Pescadores Virgen de las Mareas, por dejarme campar a mis anchas por la rula de Avilés y darme montones de muestras. También quiero agradecer la confianza (o desconocimiento) de mis supervisores, por dejarme elegir los sitios de muestreo y cómo quería hacerlo. ¡Ha sido fantástico! Y, por otra parte, también quería agradecer la colaboración involuntaria de todos los individuos que dieron la vida para formar parte de mi base de datos. Valió la pena, pues ese sacrificio ha dado buenos frutos y espero que de más y mejores.

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V.

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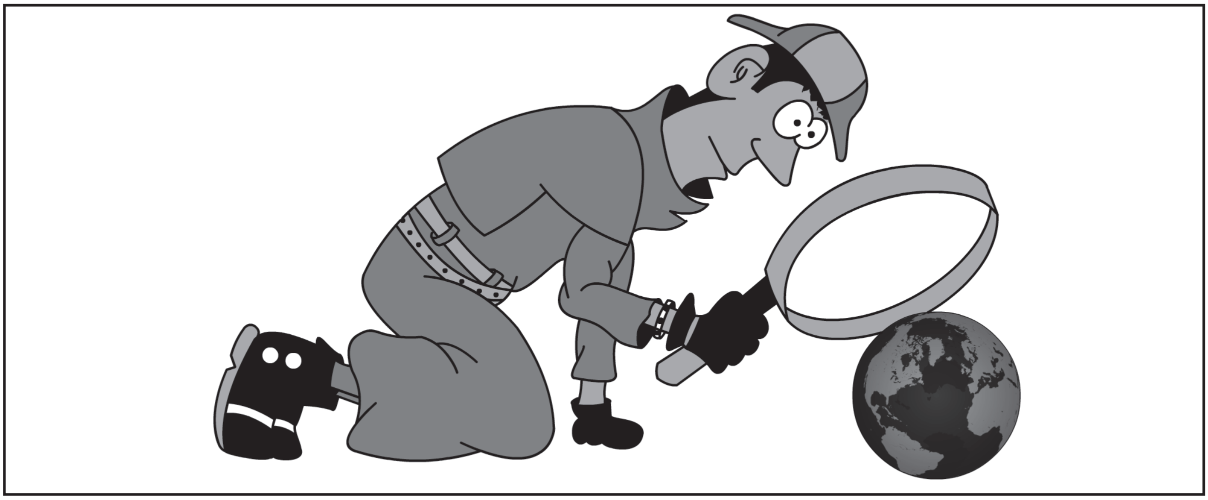
INTRODUCCIÓN



1. INTRODUCCIÓN

En el medio marino existen y conviven una gran cantidad de especies, muchas de las cuales son de gran utilidad para los humanos desde el punto de vista nutricional (Daily *et al.*, 2000). Sin embargo, al no ser el mar el hábitat natural del hombre, la visualización y el control directo de la evolución de los stocks resulta muy difícil. El estudio indirecto de estos recursos mediante diferentes métodos, incluyendo herramientas moleculares, cobra por lo tanto una especial importancia para su conservación.

En los ecosistemas naturales se considera biodiversidad al conjunto de seres vivos que coexisten de modo natural en el área de análisis, tal como queda recogido en un acuerdo establecido por la Organización de las Naciones Unidas en 1992 (Convenio sobre la Diversidad Biológica; CDB). Dentro de cada especie, los individuos presentan variaciones en su composición genética que pueden convertirse en diferencias fenotípicas (por ejemplo, distinto color o tamaño), las cuales pueden ser de utilidad al enfrentarse a distintas situaciones ambientales; es decir, pueden tener valor adaptativo.

Esta heterogeneidad de especies y variedades tanto inter como intraespecíficas conduce al establecimiento de gradientes de eficacia reproductiva que permiten que el ecosistema evolucione. El equilibrio se alcanza por la interdependencia entre los distintos eslabones de la cadena trófica, que actúa como un tampón regulando los tamaños poblacionales de depredadores y presas.

En este contexto irrumpe el ser humano, que tiene la capacidad de modificar y modifica dramáticamente el ecosistema. El hombre podría considerarse el último eslabón en la cadena trófica de los ecosistemas naturales, pero no vive en el medio marino aunque se aprovecha de sus recursos, y además no está sometido a autorregulación por la densidad poblacional de las especies explotadas. Cuando merma un sustrato trófico en un ecosistema, el humano en ocasiones cambia de lugar de explotación al igual que hacen otros predadores, pero en la mayoría de los casos intensifica las técnicas extractivas esquilmando las poblaciones y dificultando su recuperación. El hombre posee además otras características que son típicas de las especies invasoras, tal como fueron descritas en una comunicación del Consejo del Parlamento Europeo (2008): tienen la capacidad de un rápido crecimiento poblacional, alteran el área habitada sacándola del equilibrio natural, y amenazan la diversidad biológica. Los humanos extienden su influencia prácticamente a todas las especies marinas, de forma directa o indirecta.

El patrón espacial, la estructura y el funcionamiento de la mayoría de los ecosistemas están siendo alterados debido a la acción humana (Vitousek *et al.*, 1997; Sala *et al.*, 2000; Mooney y

Cleland, 2001). Muchos de los impactos con base humana perduran en el tiempo, alterando las historias evolutivas de ecosistemas y especies directa o indirectamente; es decir, tienen impactos evolutivos. Aunque hay muchos casos estudiados, dos ejemplos clásicos son muy ilustrativos. Uno es el caso de la polilla *Biston betularia*, en la cual se produjo un aumento de la frecuencia de un fenotipo oscuro en detrimento de otro más claro debido al oscurecimiento de la corteza de los árboles causado por la contaminación industrial londinense (Miller, 1999). Otro es la adaptación de los pinzones de Darwin (género *Geospiza*) a distintos tipos de alimento, tras una introducción forzada en las islas Galápagos (Boag y Grant, 1984).

Muchas actividades humanas contribuyen a cambiar el pool genético de las poblaciones al favorecer procesos como cuellos de botella, deriva génica, mutaciones y/o hibridación interespecífica entre otros, cuyos efectos pueden dejar huella a nivel evolutivo y ser irreversibles; las invasiones mediadas por el ser humano son un claro ejemplo (revisado en Mooney y Cleland, 2001). Éste y otros problemas están aumentando en las últimas décadas y, aunque se conocen sus efectos a corto plazo, su potencial huella a largo plazo no tiene aún muchas evidencias científicas (Mooney y Cleland, 2001).

Por estas razones, en el estudio de las especies y sus relaciones en el medio natural es especialmente importante analizar los efectos antropogénicos, que pueden tener efectos de muy difícil o imposible recuperación. Los estudios tienen por fuerza que estar limitados a determinadas áreas, situaciones o eventos concretos, y en función de éstos debe elegirse el material biológico adecuado para los análisis.

Las unidades biológicas en los estudios de ecosistemas son las especies y las poblaciones que las componen en una determinada área. Siguiendo la definición de Mayr (1942), se puede considerar una especie como el conjunto de individuos que comparten un acervo genético común y pueden potencialmente reproducirse entre sí. Sin embargo, todos los individuos de una especie no suelen constituir una sola entidad panmíctica (sin apareamientos preferenciales). Pese a la ausencia aparente de barreras físicas en el medio marino (Waples, 1998), las especies suelen estar divididas en poblaciones que constituyen distintas unidades reproductivas, y presentan generalmente diferencias en las frecuencias alélicas entre ellas (Wright, 1951). El aislamiento entre poblaciones no es absoluto pues suelen existir migraciones que las conectan, lo que permite que las diferencias genéticas dentro de una misma especie sean menores que entre distintas especies. Esto posibilita la identificación de especies y poblaciones mediante marcadores genéticos.

Otro aspecto a considerar es la posibilidad de que especies evolutivamente próximas entre sí coincidan en un mismo hábitat, ocasionalmente pudiendo llegar a cruzarse y producir híbridos. Generalmente, las especies que viven en simpatría presentan fuertes barreras reproductivas para mantener cada especie más o menos aislada evitando la formación de híbridos interespecíficos y la fusión de especies (Coyne y Orr, 1997; Price y Bouvier, 2002). Las barreras o mecanismos de aislamiento entre especies pueden ser tanto pre- como post-reproductivos, y abarcar desde diferente preferencia de hábitat a distinto comportamiento sexual, esterilidad o inviabilidad de los híbridos entre otros (revisado en Coyne y Orr, 1998), que impiden que especies simpátridas se fusionen, quedando aisladas reproductivamente. Sin embargo, en una situación de dificultad o estrés, estos mecanismos pueden relajarse o incluso llegar a romperse permitiendo superar ese aislamiento y dando lugar a la producción de híbridos interespecíficos (Barton, 1979).

La hibridación es una parte natural de la evolución; sin embargo, la hibridación forzada por causas antrópicas está provocando la extinción en muchos taxones debido a la introducción de alelos foráneos y el reemplazo de los alelos nativos (revisado en Allendorf *et al.*, 2001). Después de varias generaciones, el proceso puede derivar en que toda la población de un área sea de origen híbrido (enjambre híbrido o hybrid swarm; Allendorf *et al.*, 2001). Asimismo, si los híbridos son fértiles y migran a áreas cercanas, es posible que se extienda la introgresión al resto de la distribución de la especie produciéndose una profunda modificación poblacional. El conocimiento de la extensión de las zonas de hibridación e introgresión es importante para predecir el destino de las poblaciones parentales y sus híbridos (Sun y Lo, 2011), sobre todo en los casos que involucran introducción de especies no nativas que podrían amenazar a las especies locales (Blekker *et al.*, 2007).

1.1 Principales impactos humanos en el medio marino

Como se dijo anteriormente, las actividades humanas normalmente afectan al resto de especies del planeta debido a que el hombre modifica tanto los hábitats como las comunidades de seres vivos (revisado en Ramirez-Llodra *et al.*, 2011). Su impacto se ha visto amplificado principalmente a raíz de la revolución industrial (principios del S. XIX), cuando el comercio se intensificó y las fronteras entre países se hicieron menos estrictas, incrementando tanto el transporte como el intercambio de bienes y servicios por todo el planeta. Las actividades pesqueras también se industrializaron, y se ampliaron tanto las cantidades de capturas como las áreas explotadas (Pauly *et al.*, 2002). De igual manera, debido a un incremento constante en el tamaño de la población mundial (www.fao.org), las necesidades de recursos también se han visto incrementadas.

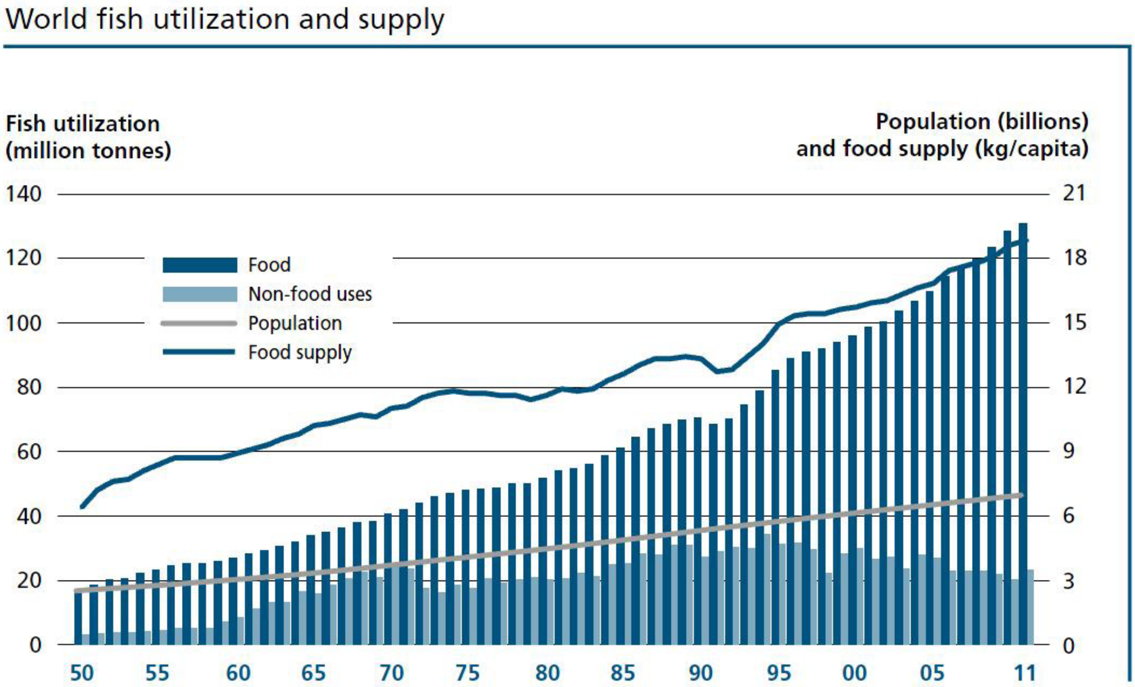
Diversos autores como de Mutsert *et al.* (2008), Polacheck (2008), Sibert *et al.* (2008) o Game *et al.* (2009) resaltan el impacto negativo de las actividades humanas en el océano y, aunque aún existe debate sobre sus efectos reales, los datos sobre descensos en el tamaño de los stocks y poblaciones de varias especies demuestran que el manejo y protección de ecosistemas y especies pelágicas es inadecuado para conservar su biodiversidad (Pauly *et al.*, 1998; Verity *et al.*, 2002; Worm y Branch, 2012). Además, un modelo propuesto por Halpern *et al.* (2008) indica que no existe ninguna zona en todo el océano que esté libre del efecto e impacto antropogénico, y que un 41% de las áreas están afectadas por múltiples factores como por ejemplo el tráfico marino, la pesca, agentes contaminantes e introducción de especies invasivas entre otros. Ese mismo estudio demuestra que los ecosistemas costeros reciben el mayor impacto acumulativo de dichos factores.

Por otra parte, las distintas especies marinas y sus poblaciones se ven afectadas en distinto grado por las actividades humanas debido a las diferencias en sensibilidad y exposición a las mismas. Analizar los efectos de un mismo factor alterante en distintos géneros y especies resulta de especial interés a la hora de comprobar si existen resultados diferenciales, si las relaciones entre ellas han sido modificadas de alguna manera, y también para identificar los grupos de organismos más sensibles a cada factor alterante y priorizar medidas de mitigación en su caso.

1.1.1 Estado y efectos de las pesquerías

Las pesquerías son quizás el factor más importante de influencia humana sobre el medio acuático, sobre todo si se considera que la presión pesquera se mantiene a lo largo del tiempo, teniendo registros de ella desde el Paleolítico superior (Utrilla-Miranda, 1981; Moure-Romanillo, 1997; Klein *et al.*, 2004 y otros). Su regulación cuantitativa está mediada por la FAO (Food and Agriculture Organization of the United Nations; www.fao.org) y por convenios internacionales que se van modificando conforme las condiciones naturales varían.

En el último informe bianual de la FAO (FAO, 2012; www.fao.org/docrep/016/i2727e/i2727e00.htm) se analiza el estado de las pesquerías en todo el mundo. Se describe un aumento sustancial en la cantidad de capturas mundiales (Figura 1). También se estima que la población humana mundial seguirá aumentando aproximadamente un 1,7% anualmente, y esto llevará previsiblemente parejo un alarmante aumento de las capturas para poder cubrir las necesidades de alimentos de calidad. En el mismo informe se indica que aproximadamente el 57% de los stocks pesqueros están al máximo nivel de explotación y un 29,9% están sobreexplotados, manteniéndose una tendencia a aumentar estos porcentajes con el paso del tiempo. Como determinaron Myers y Worm (2003), la biomasa de los stocks de pesca se reduce en un 80% en sólo 10 años de explotación. La mayoría de stocks de las diez especies más importantes, que abarcan un 30% de la producción total e incluyen especies como el atún rojo, el mero o la anguila europea, están totalmente explotados o sobreexplotados sin posibilidad de aumentar la producción pesquera (FAO, 2012).



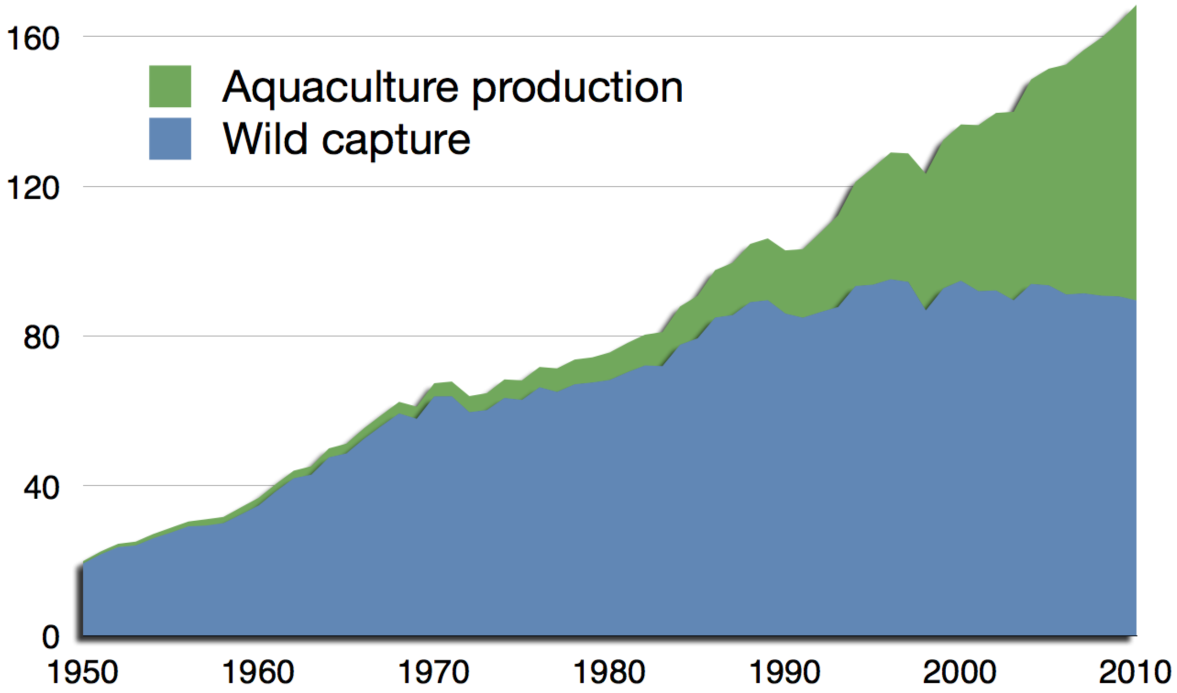
(Fig. 1 Uso de los recursos pesqueros mundiales, FAO 2012).

Para intentar conservar el recurso pesquero y controlar su explotación, la FAO (www.fao.org) recoge una serie de directrices que han sido firmadas por todos los países integrados en este organismo. Entre ellas destaca el tipo de pesca permitido (artesanal, industrial, etc.), el tamaño de red adecuado, las tallas mínimas y la cantidad de capturas (TAC, Total Allowable Catch en inglés) permitidas por cada país. En muchas especies, esta cuota de capturas o TAC viene definida basándose en el total de individuos capturados la temporada anterior y la facilidad (capturas por unidad de esfuerzo) con que se obtuvieron dichos individuos. Pero las estimas presentan varios problemas serios según indica la FAO (FAO, 2012): en primer lugar hay una discordancia en la calidad de los datos de pesca y explotación presentados por cada país, en segundo lugar se ha encontrado que más de la mitad de los países informan de manera inadecuada sobre sus capturas (en Europa, un 20,5% de los países), y en último lugar, pero no menos importante, algunas especies son difíciles de distinguir de manera visual y no son consideradas independientemente, por lo que se pescan como una unidad, impidiendo llevar a cabo un buen control de las tasas de capturas y/o explotación de cada una de ellas.

Este último problema de mala identificación se ve potenciado en los casos en que varias especies de un mismo género conviven en el mismo hábitat (simpatría), cuyas capturas se contabilizan conjuntamente como si fueran una sola especie sobre todo si son morfológicamente similares. Un ejemplo es el caso de dos especies de raya (*Dipturus cf. flossada* y *D. cf. intermedia*) descrito por Iglésias *et al.* (2004), en el que la identificación incorrecta de una de ellas (*D. cf. intermedia*) debido a una confusión taxonómica casi lleva al colapso de sus pesquerías y a la extinción de sus poblaciones. En otras ocasiones, la identificación incorrecta de las capturas se traslada al mercado, encontrándose problemas como el fraude comercial en algunas especies (Perez y Garcia-Vazquez, 2004; Woolfe y Primrose, 2004; Garcia-Vazquez *et al.*, 2011). La identificación inadecuada de capturas combinada con el aumento de la demanda pesquera puede llevar a un declive total de las especies explotadas (Pauly *et al.*, 2002).

1.1.2 Impactos de la acuicultura

Debido al hecho de que la mayoría de los caladeros pesqueros han alcanzado su máximo rendimiento desde hace años (Pauly *et al.*, 1998; Pauly *et al.*, 2002; FAO, 2012; Worm y Branch, 2012), para cubrir la demanda comercial de pescado se ha recurrido a la producción de varias especies en acuicultura (Figura 2).



(Fig. 2 Captura y producción mundial de recursos marinos, FAO 2012).
Eje Y: millones de toneladas

Este tipo de producción se ha multiplicado aproximadamente por doce en las últimas tres décadas (1980-2010) según la FAO (FAO, 2012). Actualmente se cultivan alrededor de seiscientas especies acuáticas diferentes, como por ejemplo peces (truchas, salmones, carpas, etc.), crustáceos (camarones, cangrejos, etc.) y moluscos (ostras, mejillones, etc.). En el año 2010, los moluscos representaban un 75,5% del total de producción de acuicultura (13,9 millones de toneladas; FAO, 2012). La acuicultura es capaz de producir alimento más rápido que cualquier otro medio conocido, y quizás por este hecho ha crecido tres veces más rápido que otros cultivos artificiales como la ganadería en tierra (www.fao.org). Los datos estadísticos indican que en la actualidad el 30% de los alimentos acuáticos son cultivados de este modo.

Por otra parte, las actividades de acuicultura no están repartidas por igual en todos los países. Asia, el Océano Pacífico, el África sub-Sahariana y América del sur son las áreas donde se concentra la mayor actividad mundial, habiendo llegado a convertirse en los principales productores/exportadores (FAO, 2012). En Canadá se concentra la acuicultura de mejillones, donde es una actividad de gran importancia que proporciona un 72% de la producción mundial (www.aquaculture.ca/files/species-mussels.php). Actualmente la producción se concentra principalmente en la costa este, aunque está en rápida expansión en la costa del Pacífico (www.aquaculture.ca/files/species-mussels.php).

Naylor *et al.* (2000) consideraron la acuicultura como una posible solución para preservar las poblaciones naturales de la sobrepesca al representar una fuente alternativa de especies marinas. Sin embargo, la acuicultura también tiene su lado negativo y acarrea varios problemas. En primer lugar, presenta problemas logísticos y de declaraciones irregulares de producción. Según informes de la FAO (FAO, 2012), de los 190 países que habían declarado producción, o con producción no declarada, alrededor de un 30% (incluyendo productores mayoritarios como Asia y Europa) presentaron datos incorrectos sobre la citada producción durante los años 2008-2010 (último año sobre el que se basa el documento del 2012 referenciado), con informes de escasa calidad o con vacíos temporales.

Aunque hay controversia sobre sus posibles efectos negativos, la acuicultura causa varios impactos en el medio, entre los que destacan la contaminación y degradación del hábitat debido al uso de diversos productos en las granjas (Wu, 1995; Dosdat, 2001; worldwildlife.org/industries/farmed-seafood). Un exceso de nutrientes puede llevar a su sedimentación y aumentar el crecimiento de ciertas algas, que consumen oxígeno necesario para la supervivencia de otras plantas y animales causándoles la muerte (worldwildlife.org/industries/farmed-seafood). También se genera gran cantidad de residuos fecales, como en el caso de las granjas de salmón descrito por la organización SeaWeb basándose en datos de la NOAA (www.seafoodchoices.com/resources/afishianado_pdfs/Salmon_Spring05.pdf). Además, el uso de productos químicos tales como antibióticos, pesticidas y otras sustancias tóxicas también repercute en la naturaleza causando cambios en la calidad del agua y en las comunidades que habitan cerca de las piscifactorías (Costa-Pierce, 2002; Hites *et al.*, 2004).

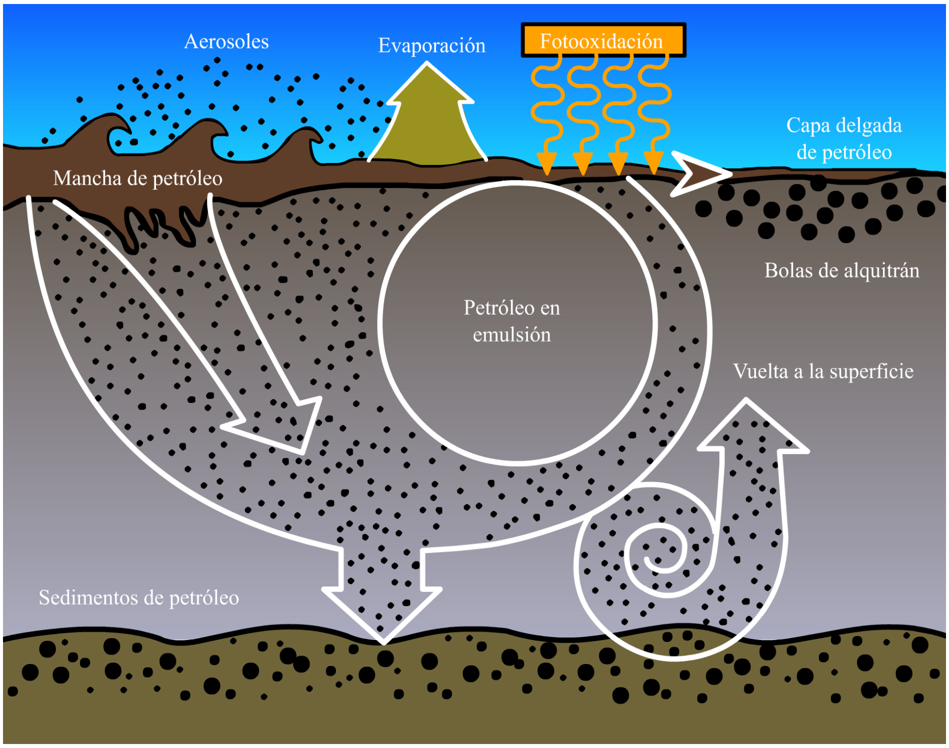
Otro problema reconocido son los escapes. La acuicultura ha ido evolucionando y adaptándose mediante nuevas tecnologías para tratar de amortiguar los distintos cambios ambientales. Sin embargo, las infraestructuras que se emplean no son muy complejas y carecen a menudo de sistemas eficaces de aislamiento y contención. Generalmente los peces se cultivan en instalaciones cerradas al medio natural (piscifactorías) y aunque en teoría los únicos intercambios con el medio ocurrirán durante el reciclado del agua, se han documentados numerosos casos de escapes accidentales que pueden poner en riesgo las poblaciones naturales vecinas, con las que interaccionan y compiten (Clifford *et al.*, 2005; Bekkevold *et al.*, 2006).

Por otra parte, en el caso de moluscos marinos como el mejillón y otros, son cultivados en el mar cerca de las costas sin prácticamente ningún impedimento para las interacciones entre los individuos cultivados en las granjas (malacolcultivos) y el medio natural. Debido a esto, los individuos cultivados y/o sus gametos o larvas pueden escaparse (Heath *et al.*, 1995) y llegar a establecerse en el hábitat natural alterando las poblaciones nativas. Estos problemas afectan tanto a los invertebrados como a los vertebrados (Naylor *et al.*, 2001, Danancher y Garcia-Vazquez, 2011), y pueden agravarse cuando se cultivan especies o razas exóticas que interaccionan con las nativas (Hindar *et al.*, 1991; Carlton, 1992; Carriker, 1992). Los individuos cultivados pueden competir por la comida (Levin *et al.*, 2001), transmitir enfermedades (McVicar, 1997) o incluso hibridar con los individuos locales (Jonsson y Jonsson, 2006). La introducción de especies exóticas en un hábitat perjudica a las especies nativas disminuyendo su abundancia (Gaston y Fuller, 2007), produciendo introgresión génica y/o desplazándolas (Anderson, 1949; Bekkevold *et al.*, 2006), induciendo cambios poblacionales (Daguin *et al.*, 2001; Braby y Somero, 2006), pérdida de biodiversidad y la aparición de zonas híbridas (Barton y Hewitt, 1985; Suchanek *et al.*, 1997; Shields *et al.*, 2010) entre otros. El efecto es más grave cuanto más continuado y prolongado sea en el tiempo.

1.1.3 Impactos a gran escala: vertidos petrolíferos

Actualmente existe otro tipo de impactos causados por el hombre cuya acción principal se produce en un corto espacio de tiempo, que tienen efectos devastadores a corto plazo pero producen también importantes secuelas que persisten en el tiempo. El caso en el que se centra esta tesis son los accidentes petrolíferos. Afortunadamente este tipo de catástrofes no son muy comunes, pero sus consecuencias son muy graves porque afectan a grandes áreas durante un largo periodo de tiempo. El petróleo constituye una de las bases del desarrollo económico y tecnológico moderno ya que es utilizado para infinidad de materiales y procesos, desde la producción de objetos plásticos hasta su uso como carburante. Como su extracción está concentrada en pocas zonas del planeta se recurre al transporte, que es la principal causa de derrames y contaminación, particularmente debido al transporte en barcos petroleros.

Una vez que el petróleo entra en contacto con el agua de mar ocurren varios procesos más o menos secuenciales que degradan parte del vertido: evaporación, disolución, dispersión, emulsificación, sedimentación, biodegradación y fotooxidación (www.cetmar.org/documentacion/comportamiento.htm; Figura 3).



(Fig. 3 Procesos degradativos del petroleo, CETMAR).

El volumen de vertido remanente varía dependiendo del tipo de fuel. Inicialmente (aproximadamente durante las primeras veinticuatro horas) se suele evaporar entre uno y dos tercios, dependiendo principalmente de la densidad del fuel, viento y temperatura del agua. La dispersión tanto química como natural (al mezclarse con el agua de mar por acción de las olas, corrientes y el viento) puede suponer la "eliminación" de otro 20% (aproximadamente; realmente se disuelve en el océano). Por último, la recogida manual del fuel que queda en superficie puede suponer la eliminación de otro 10%. Sin embargo, al mezclarse el fuel con el agua se emulsifica y su volumen puede aumentar hasta cuatro veces formando una sustancia parecida a una espuma (mousse) altamente viscosa y pegajosa. Parte del fuel forma agregados y se hunde, junto con el resto de la carga del petrolero si se hunde también, como en el caso del *Prestige*, pero muchos residuos llegan a las costas y se quedan infiltrados entre la arena y adheridos a las rocas, plantas y animales (www.cetmar.org/documentacion/comportamiento.htm).

La porción de fuel que llega a la costa causa tremendos impactos en la naturaleza. En un primer momento las especies más pequeñas, pertenecientes generalmente a un eslabón trófico inferior, junto con huevos y larvas, son las más afectadas, produciéndose en ellas una mortalidad generalizada (www.amsa.gov.au/marine_environment_protection/educational_resources_and_information/teachers/the_effects_of_oil_on_wildlife.asp). Las especies de eslabones tróficos superiores en general poseen más movilidad, pero también son afectadas gravemente por el fuel en el curso de sus movimientos de caza. Su cuerpo queda impregnado en esa mousse y se dificulta su capacidad para moverse, regular y soportar cambios de temperatura, flotar y protegerse de las infecciones entre otros efectos (ejemplo típico de un ave cubierta de fuel; Figura 4). Además el fuel ha eliminado o deteriorado sus presas, y las pocas que quedan pueden estar contaminadas y causarles una intoxicación y/o la muerte (www.cetmar.org/documentacion/comportamiento.htm; www.amsa.gov.au/marine_environment_protection/educational_resources_and_information/teachers/the_effects_of_oil_on_wildlife.asp).

(Fig. 4 Ave cubierta de fuel del *Prestige*, *The Times* 2012).

Por otra parte, los vertidos también tienen repercusión en la economía de las regiones afectadas derivada del cierre de pesquerías durante un largo período de tiempo (incluso décadas hasta su recuperación), lo que conlleva la pérdida de una gran cantidad de puestos de trabajo. Por ejemplo, en el caso del *Exxon Valdez* se destruyeron unos 26.000 puestos de trabajo y 15.000 personas perdieron su fuente de subsistencia, además de los costes económicos que supuso la limpieza de las áreas afectadas (www.noaa.gov/). El sector turístico también se ve afectado, ya que el ecosistema está totalmente alterado y degradado y las especies que allí habitaban tienen sus poblaciones mermadas. Las repercusiones económicas debidas a accidentes petrolíferos se valoran en miles de millones de euros, tal como se puede ver en los distintos casos citados por prensa internacional como la CNN o el diario *The Guardian* (money.cnn.com/galleries/2010/fortune/1005/gallery.expensive_oil_spills.fortune/index.html; www.guardian.co.uk/environment/2010/apr/30/bp-cost-deepwater-horizon-spill). En el caso del vertido del *Prestige*, un estudio realizado por la Fundación Barrie de la Maza y otras asociaciones de economistas estiman un coste aproximado de entre 3 y 5 billones de euros (Garcia, 2003).

Los efectos a largo plazo de este tipo de catástrofes son desconocidos, aunque se sabe que una exposición continuada al fuel y a los agregados que permanecen en el fondo y van liberando componentes tóxicos puede producir inmunosupresión e incluso afectar negativamente a la reproducción de diversas especies (Hooker y Gerber, 2004). Otros estudios también muestran efectos como lesiones hepáticas, aumento de radicales libres de oxígeno, neoplasmas, lesiones focales diversas, lesiones degenerativas y/o necróticas o muerte celular (Pietrapiana *et al.*, 2002 y referencias incluidas en él; www.amsa.gov.au/marine_environment_protection/educational_resources_and_information/teachers/the_effects_of_oil_on_wildlife.asp), preferentemente en especies cercanas al fondo marino y a los agregados de fuel. Considerando todo junto, es de esperar que las distintas especies y poblaciones, así como las relaciones que existan entre ellas, se vean afectadas negativamente durante un largo período de tiempo, cobrando real importancia el estudio y evaluación de los efectos de este tipo de catástrofes a largo plazo para ver cómo evolucionan en el tiempo.

1.2 Elección de especies modelo para evaluar impactos genéticos

Con el objeto de comprobar los impactos genéticos derivados de varias actividades humanas sobre especies marinas, se han elegido como modelos biológicos dos géneros marinos muy diferentes entre sí: *Lepidorhombus* (peces gallo) y *Mytilus* (mejillones). Ambos géneros poseen especies que viven en simpatría (sus distribuciones se solapan y ocupan más o menos el mismo hábitat), y por tanto se puede ver cómo pueden variar las relaciones e interacciones entre ellas y el posible efecto de los factores antrópicos considerados sobre la hibridación.

Los mejillones son animales invertebrados sésiles cuyas poblaciones ocupan un ambiente costero, mientras que los gallos son peces que ocupan zonas pelágicas y mesopelágicas. Los mejillones pertenecen a los primeros eslabones tróficos, en los cuales el tiempo de recuperación suele ser corto al poseer tasas reproductivas altas (Newel *et al.*, 1982). Por el contrario, los peces gallo pertenecen a un eslabón trófico intermedio, con tasas reproductivas más lentas (Perry *et al.*, 2005).

Lo interesante de elegir modelos de taxones evolutivos muy diferentes es que los resultados similares que se encuentren podrán considerarse generalizables. Además, el análisis de diferentes niveles tróficos es necesario para evaluar la supervivencia del ecosistema al servir de índice de sostenibilidad (Pauly *et al.*, 2002).

Los procesos de hibridación son ampliamente conocidos, afectan a organismos de todos los niveles taxonómicos y como se ha visto antes son cruciales en la evolución de las especies, la especiación y la conservación de la biodiversidad (Mallet, 2005). Para evaluar el grado de hibridación e introgresión y el estado de las poblaciones es imprescindible el uso de técnicas moleculares, ya que los híbridos a veces son imposibles de distinguir visualmente; además permiten conocer el status específico de una población al analizar unos pocos individuos (Rhymer y Simberloff, 1996; Naisbit *et al.*, 2003). En este trabajo se utilizaron marcadores diversos, ya que por la gran diferencia genómica de los grupos modelo es muy difícil elegir un tipo de genes idóneo para todos.

Como consideraciones finales, cabe reseñar que la dependencia que tiene la humanidad de los ecosistemas marinos y de las especies que en ellos habitan es de una elevada magnitud. En el océano existe una gran diversidad de especies y ejerce un papel primordial de almacén o reservorio de recursos, al formar parte del ciclo de diversos materiales y nutrientes, y para mantener el flujo de energía (Covich *et al.*, 1999; Snelgrove, 1999; Austen *et al.*, 2002; Snelgrove y Smith, 2002). La alteración del equilibrio existente en los ecosistemas (por ejemplo, disminución del número de especies y/o de individuos dentro de cada especie, o la alteración de las relaciones entre especies) afecta negativamente a la funcionalidad de los mismos (Covich *et al.*, 2004). Por ejemplo, Morin y McGrady-Steed (2004) indican que un descenso en la riqueza de especies puede alterar el funcionamiento de un ecosistema durante varias generaciones. Por ello es extremadamente importante comprender la biología, el comportamiento y cómo responden las especies de diferentes niveles tróficos y biología ante los distintos efectos producidos por las actividades humanas. Comprendiendo los efectos de las actividades humanas y su repercusión en las especies estudiadas, quizás se puedan adoptar estrategias para reducir, minimizar o incluso revertir los efectos dañinos y alteraciones que causan dichas actividades, y conseguir llegar a un punto de equilibrio entre el uso de los recursos y la preservación de la naturaleza.

2

OBJETIVOS

2. OBJETIVOS

Los objetivos generales de esta tesis doctoral son:

1. Evaluar la incidencia de la identificación incorrecta de especies simpátridas de peces gallo (género *Lepidorhombus*) en pesquerías y su posible repercusión en la conservación de los stocks.

2. Valorar el impacto de las actividades de acuicultura e introducción de especies exóticas en las poblaciones de especies nativas de mejillones (género *Mytilus*), tomando como estudio de caso una zona de acuicultura emergente como es la costa canadiense del Pacífico.

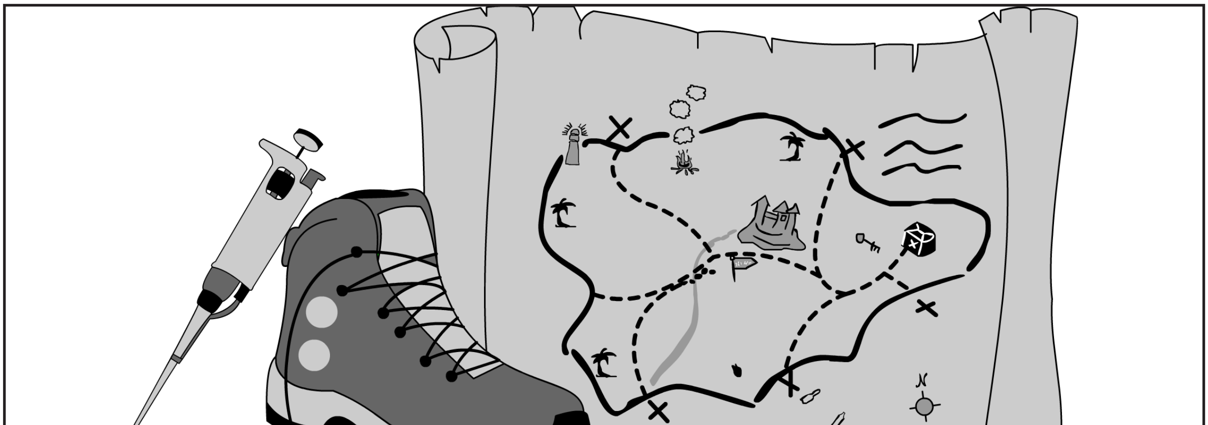
3. Estudiar el efecto del vertido petrolífero provocado por el *Prestige* en las relaciones interespecíficas de las dos especies simpátridas de peces gallo (género *Lepidorhombus*).

4. Determinar los cambios en la estructura poblacional detectables en el género *Lepidorhombus* diez años después del accidente del *Prestige*.

5. Analizar los efectos del *Prestige* en las comunidades de mejillones (género *Mytilus*) del norte de España a nivel de diversidad poblacional e introgresión interespecífica.

3

MATERIAL Y MÉTODOS



3. MATERIAL Y MÉTODOS

En este apartado se describe de forma general el tipo de muestras analizadas, su procedencia y las distintas técnicas empleadas para su análisis. Posteriormente, en cada capítulo del apartado *Resultados* dentro de la correspondiente sección de *Material y métodos* (Material and methods) se especificarán de manera más detallada, así como los análisis estadísticos requeridos en cada caso.

3.1 - Especies de estudio

Como material biológico se utilizaron individuos de los géneros *Lepidorhombus* y *Mytilus*, que presentan diferencias en sus características específicas y poblacionales, en su distribución geográfica y en la capacidad de movilidad y dispersión de sus individuos. Además engloban diferente número de especies.

3.1.1 - Género Lepidorhombus

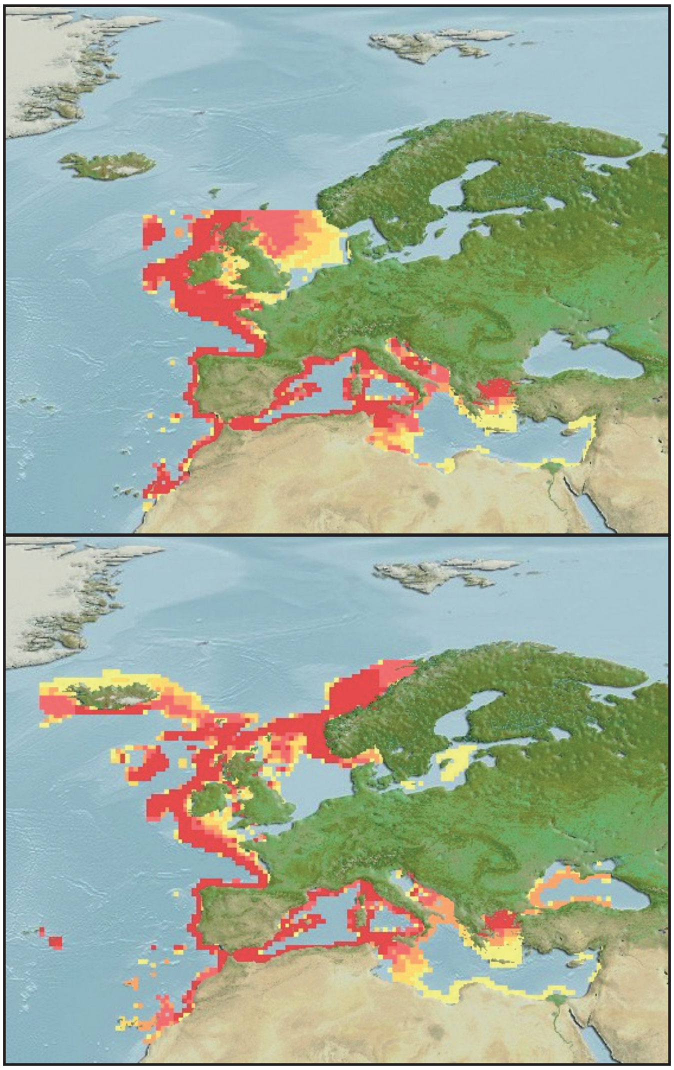
3.1.1.1 – Distribución y aspectos generales

El género *Lepidorhombus* se compone de dos especies demersales: El gallo de cuatro manchas o *Lepidorhombus boscii* (Risso, 1810) y su congénere el gallo común o *L. whiffiagonis* (Walbaum, 1792) (Figura 5). Dichas especies pertenecen a la familia de los Scophthalmidae, que se engloba a su vez dentro del orden de los Pleuronectiformes y ambos son comúnmente denominados como gallos (megrim en inglés), aunque suelen tener nombres particulares típicos de cada región tales como bruixa, rapante u ojito en España. Son peces planos y tienen los dos ojos en el lado izquierdo del cuerpo, separados por una prominencia ósea. Los ojos se encuentran muy juntos, y el superior (que durante el desarrollo migra desde el lado del cuerpo que quedará hacia el fondo marino) se encuentra ligeramente retrasado respecto al ojo inferior. Ambas especies se distribuyen en el noreste del océano Atlántico y en el mar Mediterráneo solapando en prácticamente todo su territorio (Figura 6). Además, también presentan patrones ecológicos, biológicos y características morfológicas muy similares (Nielsen, 1990), explicados más detalladamente dentro de los correspondientes capítulos del apartado *Resultados*.

(Fig. 5 Especies que componen el género *Lepidorhombus*: *L.boscii* (izquierda) y *L. whiffiagonis* (derecha))

3.1.1.2 – Interés pesquero

Ambas especies son de un elevado interés comercial para los países europeos y son pescadas mediante técnicas de arrastre o deriva en áreas de pesca mixta junto con otras especies como la merluza, el rape y la cigala (SeaFish species guide 2012; www.seafish.org). En los controles pesqueros no suele hacerse distinción alguna entre las dos especies, manejándose por tanto como una sola unidad (ICES WGHMM, 2008). Por ello tienen una cuota de capturas o TAC conjunta y, como es definida basándose en el total de capturas pescadas el año anterior, ello hace imposible un buen control sostenible de los niveles de explotación. España es el principal país pescador de gallos (31,86% en el año 2010), seguido de Reino Unido, Francia e Irlanda, capturando entre estos cuatro países un 95,84% del total (www.fao.org/figis/servlet/SQServlet?file=/work/FIGIS/prod/webapps/figis/temp/hqp_2621341219924161137.xml&outtype=html).



(Fig. 6 Distribución de las poblaciones de las especies de gallo, *Lepidorhombus boscii* (arriba) y *L. whiffiagonis* (abajo), www.fishbase.org)

3.1.1.3 – Estructura poblacional

Respecto a la estructuración poblacional, las dos especies de gallo parecen tener patrones relativamente distintos. Por una parte, *L. whiffiagonis* presenta bastante homogeneidad entre sus poblaciones del océano Atlántico y éstas difieren genéticamente de las del Mediterráneo. Mientras que por otro lado, *L. boscii* sigue un patrón de aislamiento por distancia a lo largo de las aguas del Atlántico y Mediterráneo (Danancher y Garcia-Vazquez, 2009). Hasta la fecha sólo se han estudiado las especies por separado y no en conjunto analizando sus posibles interacciones.

Su historia evolutiva ha sido trazada basándose en secuencias mitocondriales, indicando que las dos especies divergieron hace aproximadamente 6,75-5,84 millones de años (Campo y Garcia-Vazquez, 2010), quedando aislado *L. boscii* en el océano Atlántico y *L. whiffiagonis* en el mar Mediterráneo. Más tarde, en torno a hace 5,33 millones de años, las dos especies volvieron a encontrarse entrando en contacto secundario tras la reapertura del estrecho de Gibraltar (Campo y Garcia-Vazquez, 2010).

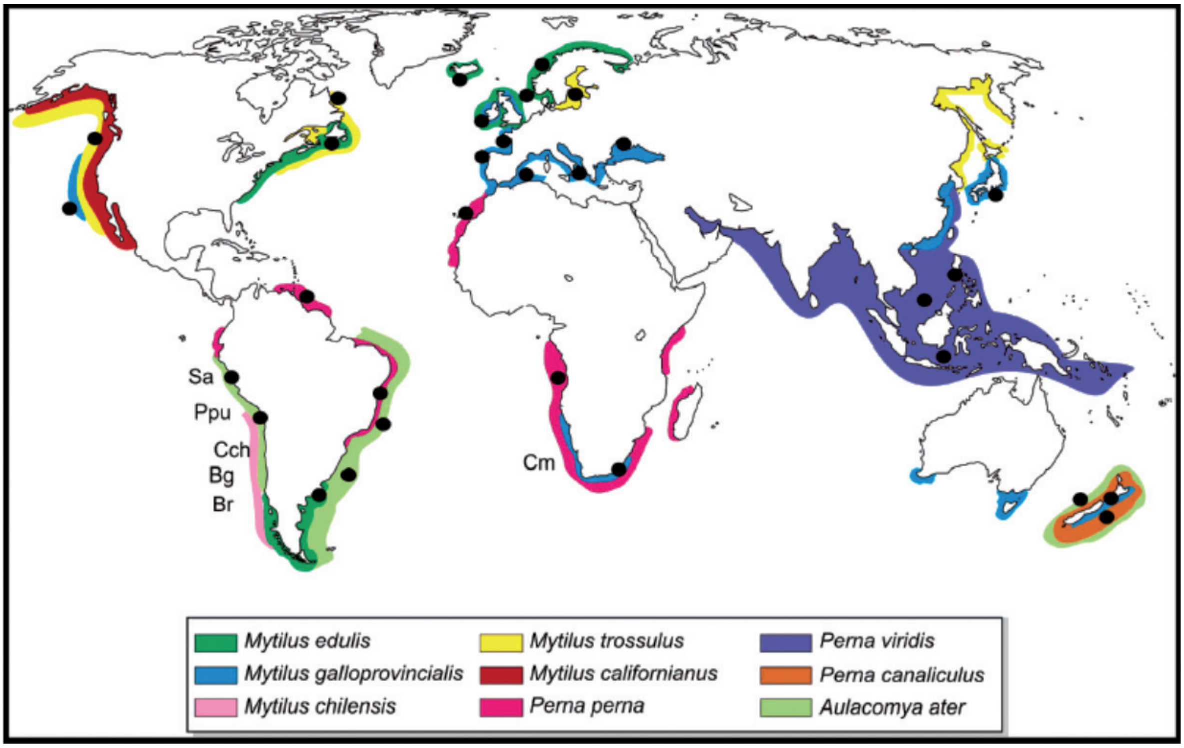
3.1.2 - Género Mytilus

3.1.2.1 – Distribución y aspectos generales

El género *Mytilus* se compone de seis especies, de las cuales en esta tesis se han estudiado las siguientes cuatro: *Mytilus edulis* (Lineo, 1758), *M. galloprovincialis* (Lamarck, 1819), *M. californianus* (Conrad, 1837) y *M. trossulus* (Gold, 1850) (Figura 7). Tradicionalmente, las

(Fig. 7 Especies que componen el género *Mytilus*: *M. edulis* (arriba izquierda), *M. galloprovincialis* (arriba derecha), *M. californianus* (abajo izquierda) *y M. trossulus* (abajo derecha). Fotografías de L. Schroeder).

especies *Mytilus edulis*, *M. galloprovincialis* y *M. trossulus* se engloban dentro de un grupo denominado *complejo de Mytilus edulis* ("*The Mytilus edulis complex*"; Gosling, 1992a) debido a su similitud en la morfología de la concha, a veces influenciada por el ambiente en el que crecen (Inoue *et al.*, 1995). El género *Mytilus* se agrupa dentro de la familia Mytilidae, que pertenece al orden Mytiloida. Todas ellas son especies de moluscos cosmopolitas y se conocen popularmente con el nombre de mejillones (mussels en inglés). Los individuos pertenecientes a estas especies poseen una serie de características comunes, como la presencia de una concha con dos valvas compuestas de carbonato cálcico (de morfología más o menos diferente en cada especie) unidas entre sí mediante un ligamento con función de bisagra (charnela). La concha bivalva protege el cuerpo, que es blando y suele ser de color anaranjado y con los bordes de color negro. Los mejillones son animales sésiles filtradores que se establecen generalmente fijándose a las rocas con sus filamentos (de naturaleza proteica) producidos en la glándula de biso, formando grupos de individuos a veces bastante numerosos en la zona intermareal baja y media expuesta a las olas, donde constituyen una parte muy importante de la comunidad (Koehn, 1991; Suchanek, 1985).



(Fig. 8 Distribución de las especies de mejillón en el mundo; figura extraída de Santaclara *et al.*, 2006).

Las distintas especies ocupan hábitats parcialmente distintos (Figura 8), y generalmente viven en aguas templadas o frías y limpias, lo cual es interesante ya que se pueden usar estas especies como indicadoras de contaminación medioambiental. Estos animales poseen una fecundación externa en la que tanto el esperma como los óvulos se liberan al medio acuático. Tras la fecundación, las larvas se pueden mantener en una columna de agua varias semanas o incluso meses (Bayne, 1965; Suchanek, 1985) hasta que llegan a la costa y se establecen en una zona generalmente rocosa. El aparato respiratorio que poseen estos bivalvos es de tipo branquial en el que las branquias se usan tanto para obtener el oxígeno del agua como para filtrar y seleccionar las pequeñas partículas que componen su alimento (principalmente fitoplancton y materia orgánica en suspensión).

3.1.2.2 – Interés a nivel de producción

Entre los muchos intereses que pueden tener estas especies de mejillón (además del medioambiental antes mencionado) también poseen un alto interés como alimento. En la actualidad, todas las especies consideradas en esta tesis son cultivadas artificialmente en granjas de acuicultura.

El análisis de producción por países resulta complicado al carecer de datos de gran parte de ellos. Por ejemplo, para Canadá o el resto de países americanos no hay datos concretos aunque se estima una alta tasa de producción. La FAO también estima, por ejemplo, que China sería otro país con gran producción de *Mytilus galloprovincialis* (estimado en aprox. 450.000 toneladas anuales; www.fao.org), seguido de cerca por España (estimado en aprox. 250.000 toneladas anuales, www.fao.org; cultivados principalmente en las rías gallegas) y a continuación se encuentran otros países como Italia y Francia.

Globalmente hay una larga tradición en lo que se refiere al consumo de mejillones y otros moluscos. Por ejemplo, en la costa cantábrica se encuentran restos de grandes depósitos de conchas denominados "conchales" pertenecientes a períodos arqueológicos muy antiguos tales como finales del Paleolítico superior o el Mesolítico (*i.e.*, hace más de 6.000 años; Utrilla-Miranda, 1981; Moure-Romanillo, 1997). A partir del siglo XX se empiezan a cultivar a gran escala (Figueras, 1989) fijando las semillas de mejillón a postes establecidos cerca de la orilla del mar, simulando su hábitat natural donde reciben el efecto de la marea. Más tarde se desechó este método y se optó por el cultivo en unas estructuras flotantes de las que cuelgan una especie de cuerdas a las que se fijan los mejillones. Mediante estas técnicas, los mejillones están sujetos a la depredación por parte de estrellas de mar, peces y diversas aves al estar expuestos al medio natural, pero las pérdidas ocasionadas parecen no ser significativas.

3.1.2.3 – Estructura poblacional

El patrón de diferenciación genética poblacional entre las especies de mejillones se conoce relativamente bien, ya que son uno de los géneros de moluscos más estudiados y se dispone de una gran variedad de marcadores para la identificación de las especies, que van desde alozimas (McDonald *et al.*, 1991), a la subunidad 18S del ADN ribosómico nuclear (rDNA) (Kenchington *et al.*, 1995) y otros marcadores nucleares especie-específicos (por ejemplo, Rawson *et al.*, 1996). Sin embargo, aún no se ha encontrado una clara estructura relacional interespecífica basándose tanto en su filogenia como en su clasificación a partir de marcadores genéticos; aunque parece claro que las especies del *complejo de Mytilus edulis* están más próximas entre sí que el resto de las especies del género (Kenchington *et al.*, 1995; Santaclara *et al.*, 2006). Tampoco tienen una estructura poblacional claramente definida. Esto puede ser quizás debido a su alta capacidad dispersiva (siendo larvas pueden desplazarse entre 30 y 60 Km, pero en algunos casos se han descrito desplazamientos de más de 100 Km; McQuaid y Phillips, 2000; Gilg y Hilbish, 2003) y la esperada panmixia entre las poblaciones costeras (Shields *et al.*, 2010). La hibridación entre las distintas especies de mejillones del *complejo de Mytilus edulis* es un hecho comprobado y se considera normal debido al hecho de compartir tanto hábitat como linaje (Gosling, 1992b), lo cual se constata con las numerosas publicaciones haciendo referencia a dichas hibridaciones interespecíficas (por ejemplo, Gosling, 1992b; Heath *et al.*, 1995; Suchaneck *et al.*, 1997; Hilbish *et al.*, 2002; Wood *et al.*, 2003; Miranda *et al.*, 2010).

3.2 - Zonas de estudio

El objetivo general de esta tesis se centra principalmente en estudiar una serie de impactos causados esencialmente por la acción humana. Concretamente se eligieron tres situaciones distintas: el impacto causado por una mala identificación de los individuos pescados, el impacto causado por granjas de acuicultura y el impacto provocado por el accidente del petrolero *Prestige*. Para su estudio se eligieron distintas poblaciones de los dos géneros considerados previamente (*Lepidorhombus* y *Mytilus*) acordes con su distribución y su relación con las áreas afectadas.

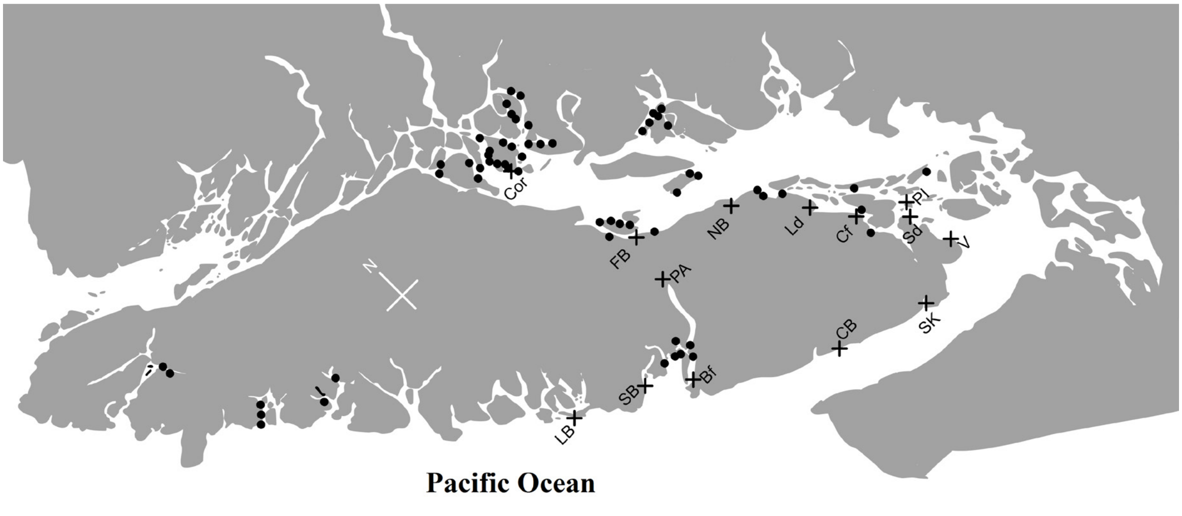
3.2.1 - Identificación errónea de especies

Tal y como se dijo anteriormente, las dos especies de peces gallo (género *Lepidorhombus*) son pescadas conjuntamente. La mayoría de minoristas rotulan ambas especies como *Lepidorhombus spp.* o no hacen distinción entre ambas identificándolas como una sola especie. Se llevó a cabo un estudio de mercado en el que se evaluó el correcto etiquetado en ambas especies de gallo por todo el territorio español para comprobar si era un fenómeno generalizado. Para realizar esta tarea, se recogieron muestras de distintos supermercados y rulas de Galicia, Asturias, Madrid, Cataluña y Valencia, cubriendo por tanto parte de la distribución atlántica y mediterránea de estas especies.

3.2.2 - Impacto de las granjas de acuicultura.

Para analizar el posible efecto de las granjas de acuicultura sobre las relaciones interespecíficas en poblaciones naturales, se estudiaron granjas de mejillones de la isla de Vancouver (Columbia Británica, Canadá; Figura 9). La elección de este emplazamiento es debido a que en esta zona se cultivan varias especies de mejillón a gran escala. Aunque también se cultivan en Galicia, esta zona ha sido afectada por la marea negra del *Prestige* (que se estudiará en otro apartado de esta memoria) y las posibles interacciones entre impactos podrían hacer muy difícil identificar los efectos de la acuicultura por separado, por lo que no se considerará como estudio de caso.

La isla de Vancouver está situada en la costa oeste de Canadá y posee una gran abundancia de granjas de mejillones, donde se cultivan tanto la especie nativa *Mytilus trossulus* como las no nativas *M. edulis* y *M. galloprovincialis*. Dichas granjas no están distribuidas homogéneamente en todo el territorio insular, no cultivan las mismas especies y los distintos puntos muestreados poseen distintas características en cuanto a abundancia de granjas y facilidad de dispersión de larvas e individuos debido a diferencias en las corrientes marinas existentes.

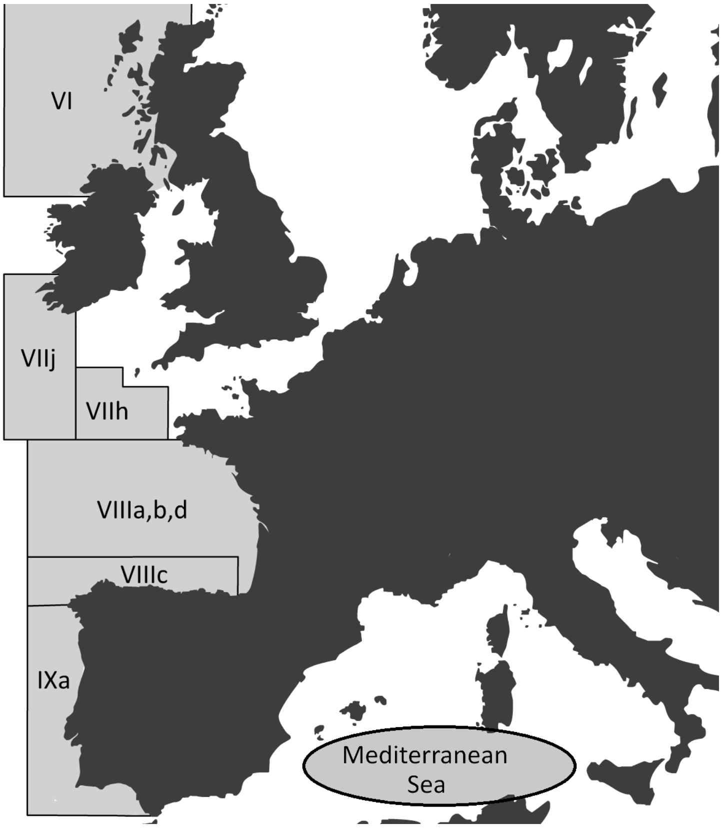


(Fig. 9 Mapa de la isla de Vancouver (Canadá) y localización de las granjas de acuicultura de mejillón (círculos) y los puntos de muestreo (cruces))

3.2.3 - Impacto del Prestige

El petrolero *Prestige* se hundió enfrente de las costas gallegas el 19 de Noviembre de 2002, y el vertido afectó a toda la zona norte de la Península Ibérica (cornisa Cantábrica) desde Galicia hasta la ría de Arcachon (Francia) y las aguas situadas enfrente de este territorio. Los efectos que causó dicho vertido petrolífero fueron estudiados en los dos géneros considerados previamente (*Lepidorhombus* y *Mytilus*).

- En el caso del género *Lepidorhombus* se estudiaron poblaciones que cubrieron tanto áreas afectadas como no afectadas. Las muestras se tomaron en varios años e incluyen tanto muestras previas al accidente como otras de varias generaciones posteriores, conteniendo individuos de ambas especies. Los puntos muestreados cubren la distribución de ambas especies y engloban las áreas denominadas por el ICES (International Council for the Exploration of the Sea) VIIIc, VIIIa,b,d, VIIj, VIIh y VI, así como del mar Mediterráneo (Figura 10).



(Fig. 10 Mapa de Europa y localización de las áreas muestreadas (gris claro))

- En el caso del género *Mytilus*, se muestrearon varias áreas rocosas del litoral Cantábrico que cubrieron el área afectada desde las costas gallegas hasta Cantabria. Se muestrearon zonas intermareales de playas rocosas de Ortigueira, Figueras, Porcia, Luarca, San Pedro, Gijón, Colunga, Toró y La Franca (Figura 11), y también se incluyen muestras de antes y después del accidente.

(Fig. 11 Mapa del norte de España y localización de los puntos muestreados (cruces))

3.3 - Tipo de muestreo y obtención de tejido.

En el caso de los gallos, se obtuvieron muestras tanto de individuos adultos como de juveniles. Los especímenes se obtuvieron durante distintas campañas oceanográficas durante el proyecto europeo MARINEGGS (QLK5-CT1999-01157, 5º Programa Marco); en cruceros científicos del NAFC Marine Centre (University of the Highlands and Islands, Escocia), y también contactando con diversas rulas, pescadores y distribuidores o comprándolos directamente en supermercados. A partir de los individuos obtenidos se diseccionó una fracción de tejido branquial o muscular (aprox. 4 cm^3) para su posterior análisis y se conservó a temperatura ambiente en tubos rellenos de etanol al 95% hasta la extracción de ADN.

En el caso de los mejillones, sólo se tomaron muestras de individuos adultos. Todos los especímenes se obtuvieron mediante muestreo manual en las zonas rocosas de las localizaciones previamente mencionadas. Las muestras se conservaron a temperatura ambiente en tubos rellenos de etanol al 95% hasta la extracción de ADN.

La cantidad y tipo de muestras empleadas se describirá en cada subapartado de la sección de Resultados de la presente tesis.

3.4 - Extracción de ADN

A partir de los fragmentos de tejido previamente descritos conservados en etanol, se procedió a la extracción del ADN total de los individuos mediante dos procedimientos diferentes según las muestras:

- En el caso de los mejillones obtenidos en la isla de Vancouver, el procedimiento de extracción de ADN consistió en diseccionar un fragmento del músculo del pie (aprox. 50 mg) y disgregar el tejido en un tubo que contenía entre 8 y 10 bolas de sílice de 1mm de diámetro mediante fricción usando un agitador de tubos. Posteriormente se añadieron 80 µL de reactivo PrepManTM Ultra (Applied Biosystems) siguiendo las instrucciones del fabricante y se incubaron en un horno a 100ºC durante 15 minutos. Posteriormente se dejaron enfriar las muestras y el sobrenadante, que contenía el ADN, fue transferido a tubos nuevos. El ADN de las muestras fue conservado a -20ºC.

- En el caso del resto de muestras (mejillones procedentes de España y muestras de pez gallo) la extracción de ADN se realizó empleando la resina Chelex® (Bio-Rad Laboratories) y siguiendo el protocolo descrito en Estoup *et al.* (1996). Este procedimiento consiste en diseccionar un fragmento de tejido (aprox. 50 mg) y situarlo en un tubo al cual se añade a continuación 500 µL de una solución de Chelex al 12.5% (peso/volumen) y 7,5 µL de proteinasa K (20 mg/mL). A continuación se incuban las muestras durante hora y media a 56º, agitándolas con un vórtex cada 15 minutos. Finalmente se procede a inactivar la proteinasa K mediante incubación de las muestras en un horno a 100ºC durante 20 minutos. El ADN queda en el sobrenadante, mientras que la resina precipita en el fondo del tubo. Las muestras se conservaron a corto plazo a 4ºC y a largo plazo a -20ºC.

3.5 - Amplificación de ADN

Para la realización de esta tesis se amplificaron varios fragmentos de ADN mediante la técnica de amplificación por reacción en cadena de la polimerasa (PCR). La elección de los marcadores genéticos más adecuados para cada tipo de estudio genético se llevó a cabo teniendo en cuenta las características genéticas y evolutivas propias de cada marcador, así como sus niveles de variabilidad genética en las muestras objeto de estudio, tal como sugiere Carvalho (1998). En este trabajo se han empleado tanto marcadores nucleares como mitocondriales:

- Loci microsatélite: Los loci microsatélite también se conocen como repeticiones cortas en tándem (short tandem repeats en inglés, STR) y se caracterizan por ser repeticiones de secuencias de entre una y seis bases de ADN entre cinco y cuarenta veces, aunque mayores repeticiones también son posibles (Selkoe y Toonen, 2006). Debido a que poseen herencia mendeliana simple, son marcadores neutrales, co-dominantes, muy polimórficos, cada alelo puede ser fácilmente identificado y han demostrado ser muy versátiles para múltiples estudios. Estos marcadores se consideran herramientas muy apropiadas para estudios de ecología y de análisis de poblaciones (Jarne y Lagoda, 1996; revisado en Selkoe y Toonen, 2006). Además, debido a su elevado polimorfismo, son capaces de detectar patrones de diferenciación poblacional débiles incluso en especies con un gran flujo génico (Waples, 1998).

  Un total de seis loci microsatélite fueron empleados en los estudios realizados en gallos: Lepi-P8, Lepi-P21, Lepi-P29, Lepi-P34, Lepi-P38 y Lepi-P40 (Danancher y Garcia-Vazquez, 2009).

- Gen del 5S ARNr: El ARN ribosomal 5S es un componente integral de la subunidad grande de los ribosomas ampliamente estudiado en muchos organismos y que se encarga de intensificar la síntesis de proteínas (Szymanski *et al.*, 2002). En los genomas de eucariotas, está organizado como una unidad estructural compuesta de dos partes (región codificante y espaciador no transcribible) repetidas en tándem varias veces. Debido a su elevada importancia funcional, la región codificadora presenta una baja tasa de sustitución manteniéndose muy conservada tanto en secuencia como en longitud a lo largo de la evolución, siendo similar entre todos los organismos (Hori y Osawa, 1987). Por otra parte, el espaciador no transcribible (o non-transcribed spacer, NTS en inglés) no es funcional y se cree que es neutral ante la selección, y por lo tanto es susceptible de acumular mutaciones y variar en longitud entre diferentes especies (Garcia-Vazquez *et al.*, 2009). Debido a estas características, se han empleado las diferencias en longitud del NTS como marcador específico para identificar las distintas especies estudiadas de gallo.

- Secuencia del D-loop mitocondrial: El D-loop es una región no codificante que forma parte de la región control del ADN mitocondrial (ADNm) y contiene los principales elementos reguladores para la replicación y expresión del ADNm (Sbisà *et al.*, 1997). Numerosos estudios señalan que esta región (D-loop) es la parte del ADNm que más rápido evoluciona (Saccone *et al.*, 1993) y, debido al conocimiento que hay sobre ella, resulta un buen modelo para estudiar procesos evolutivos entre especies (Sbisà *et al.*, 1997). En la secuencia del D-loop mitocondrial pueden variar algunos nucleótidos entre individuos de la misma especie, pero entre distintas especies los cambios son mayores permitiendo tanto identificar la especie de pertenencia de un individuo como realizar estudios poblacionales.

- Locus Glu-5': Este gen codifica para una proteína polifenólica de mejillón. Dicha proteína es producida por una glándula endocrina situada en el pie del mejillón y es un componente clave en el establecimiento y sujeción del mejillón al sustrato (Waite, 1992). Este marcador fue descrito por Rawson *et al.* (1996) y permite diferenciar las tres especies pertenecientes al *complejo de Mytilus edulis* (*M. edulis*, *M. galloprovincialis* y *M. trossulus*) ya que el fragmento amplificado posee diferentes tamaños en las diferentes especies y son fácilmente identificables en un gel de agarosa tras la amplificación mediante PCR (Rawson *et al.*, 1996).

- Locus Me: Este gen codifica para una proteína adhesiva del pie del mejillón denominada Proteína del pie 1. Esta proteína tiene dominios repetitivos y no repetitivos y es capaz de diferenciar las tres especies pertenecientes al *complejo de Mytilus edulis* (*M. edulis*, *M. galloprovincialis* y *M. trossulus*), por diferencias de tamaño en la región no repetitiva codificante (Inoue *et al.*, 1995).

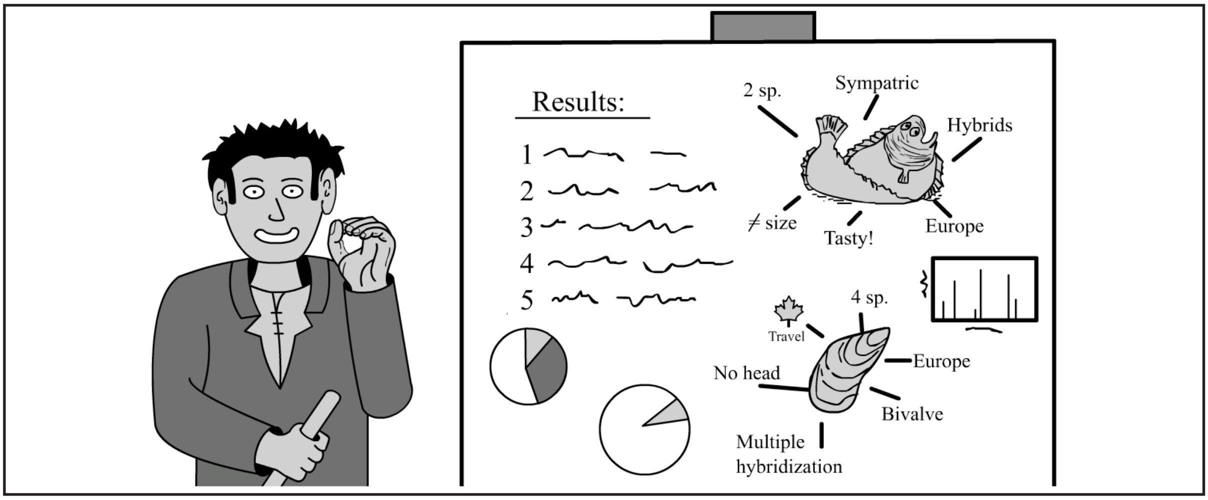
- Gen de la subunidad 1 de la citocromo oxidasa c (COI): Este gen mitocondrial codifica la subunidad 1 de la última enzima de la cadena respiratoria mitocondrial, encargada de convertir una molécula de oxígeno en dos de agua. El COI se caracteriza por tener una tasa de sustitución relativamente alta, aunque varía en función del organismo considerado. Además, posee una secuencia similar entre los individuos de una misma especie variando entre distintas especies. Debido a estas características, este gen se ha utilizado ampliamente en estudios filogenéticos y poblacionales (por ejemplo, Hoeh *et al.*, 1996; Campo y Garcia-Vazquez, 2010). Además, el gen COI se usa en la iniciativa Barcoding (ver por ejemplo Hebert y Gregory, 2005), creada en 2003 con la finalidad de desarrollar una base de datos global para la identificación de especies mediante unos pocos marcadores.

A menudo los estudios basados en marcadores nucleares y mitocondriales llevan a las mismas conclusiones (Perdices *et al.*, 2001); sin embargo, en ocasiones hay cierta discrepancia y esto permite identificar diferenciación a pequeña escala o discordancia entre patrones genéticos (Monsen y Blouin, 2003). La utilización conjunta de marcadores nucleares y mitocondriales, multiplica la efectividad de estas herramientas en estudios poblacionales (Brunner *et al.*, 1998).

Adicionalmente, en cada capítulo de la sección de resultados se detallará la metodología correspondiente utilizada.

4

RESULTADOS



4. RESULTADOS

Lista de publicaciones:

Los resultados obtenidos en la presente tesis doctoral han dado lugar a las siguientes publicaciones en revistas científicas de impacto internacional indexadas en el SCI y componen los distintos capítulos de la sección de *Resultados*:

- Capítulo 1: **Crego-Prieto V**, Campo D, Perez J, Martinez JL, Garcia-Vazquez E, Roca A (2012). Inaccurate labelling detected at landings and markets: The case of European megrims. Fisheries Research 129-130, 106-109.

- Capítulo 2: **Crego-Prieto V,** Juanes F, Roca A, Taylor JS, Garcia-Vazquez E (2013). Aquaculture as a gate for invasions. Hybrid zones of invasive NIS and native *Mytilus* mussels are linked with farms in Vancouver Island. (Under revision in Biological Invasions).

- Capítulo 3: **Crego-Prieto V**, Martinez JL, Roca A, Garcia-Vazquez E (2012). Interspecific Hybridization Increased in Congeneric Flatfishes after the *Prestige* Oil Spill. PLoS ONE 7(4): e34485. doi:10.1371/journal.pone.0034485.

- Capítulo 4: **Crego-Prieto V,** Danancher D, Campo D, Roca A, Garcia-Vazquez E (2013). Interspecific introgression and changes in population structure in a flatfish species complex after the *Prestige* accident. Marine Pollution Bulletin 74, 42-49. doi: 10.1016/j.marpolbul.2013.07.035.

- Capítulo 5: **Crego-Prieto V**, Arrojo-Fernández J, Prado A, Machado-Schiaffino G, Roca A, Garcia-Vazquez E (2013). Cytological and population genetic changes in Northwestern Iberian mussels after the *Prestige* oil spill. (Under revision in Estuaries and Coasts).

Fisheries Research

Inaccurate labelling detected at landings and markets: The case of European megrims

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Abstract

Many marine stocks are endangered by inadequate or unreported exploitation, and mislabelling can enhance the problem. We focused this work on two morphologically similar marine species that are caught together in mixed fisheries, the European common megrim (*Lepidorhombus whiffiagonis*) and the four-spotted megrim (*L. boscii*). We sampled 239 megrim individuals directly from landings from five different points of Spain, the world's main consumer of megrim, and 264 megrims from five different markets. Samples were genetically analyzed and identified with a species-specific marker. We found that 40% of megrims were incorrectly labelled at landings, and this mislabelling error increased to 60% at markets, the highest values ever reported for fish and shellfish. In addition, although most landings across Europe (90%) were identified as *L. whiffiagonis*, genetic results showed that only 49% belonged to this species and the rest were *L. boscii* suggesting a unidirectional mislabelling. Erroneous identification of fish catches leads to inaccurate estimates of exploitation and, if prolonged over time, can contribute to the exhaustion of stocks and loss of genetic diversity. Our results highlight the urgency of a separate management plan for each megrim species in markets and the need to apply currently available molecular markers if visual methods are not good enough for species identification.

Keywords: Flatfish; Fisheries; Mislabelling; *Lepidorhombus boscii*; *Lepidorhombus whiffiagonis*.

Citation: Crego-Prieto V, Campo D, Perez J, Martínez JL, Garcia-Vazquez E, Roca A (2012). Inaccurate labelling detected at landings and markets. The case of European megrims. Fisheries Research, 129-130, 106-109. DOI: 10.1016/j.fishres.2012.06.017

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1. INTRODUCTION

Stocks of many aquatic species become endangered (Hutchings 2000) due to the increasing need for food while humankind continues growing (www.fao.org). Some examples are sharks (Baum *et al.* 2003; www.iucnredlist.org) and bluefin tuna (Safina and Klinger 2008). In 2008, the Food and Agriculture Organisation (FAO) in its biannual report (www.fao.org/docrep/011/i0250e/i0250e00.htm) estimated that half of the world's fisheries are at maximum exploitation and another quarter are over-exploited or depleted (as also is revealed for example in Myers and Worm 2003). A recovery trend was shown in the 2010 FAO report (www.fao.org/docrep/013/i1820e/i1820e.pdf) for some stocks due to improvements in management (*e.g.* blue whiting, Arctic cod or Greenland and Atlantic halibut), but many other species (*e.g.* different tuna species, Atlantic cod, and some hake species) remain overexploitated.

Illegal and unreported fishing occurs in a large number of species (Agnew *et al.* 2009) and certainly contributes to overexploitation. However a solution is not easy and requires internationally concerted efforts for monitoring fishing grounds (Sumaila *et al.* 2006). Besides IUU (illegal, unreported and unregulated) fishing, other factors that can lead to inadvertent overfishing include the inaccurate estimation of exploitation rates, which may represent a major obstacle for developing sustainable management guidelines. Mislabelling is one of the factors involved in underreported exploitation of stocks and could threaten the sustainability of fisheries contributing to the depletion of fishery resources as occurs for example in the Patagonian toothfish *Dissostichus eleginoides* (Marko *et al.* 2011), the European common skate *Dipturus batis* (Iglésias *et al.* 2010), and different hake species (Garcia-Vazquez *et al.* 2012). Incorrect identification of fish species causes incorrect quantification of catch at landings and consequently leads to inaccurate estimates of exploitation rates. Taxonomic confusion is more likely when two species are morphologically similar, like the above cited common skate, which actually are two different species, the blue skate (provisionally called *D. cf. flossada*) and the flapper skate (*D. cf. intermedia*; Iglésias *et al.* 2010), some sharks (Clarke *et al.* 2006, Boomer *et al.* 2010), North American (Garcia-Vazquez *et al.* 2009) and African hakes (Garcia-Vazquez *et al.* 2012) and others. The problem can be severe and can place underreported species on the verge of extinction (*e.g.* Griffiths *et al.* 2010).

In this work, we considered the megrims *Lepidorhombus boscii* (four-spotted megrim, Risso 1810) and *L. whiffiagonis* (common megrim, Walbaum 1792) which are two morphologically similar flatfish species belonging to the Scophthalmidae family (Pleuronectiformes), and economically important for European fisheries (*e.g.* Pinnegar *et al.* 2002). *L. whiffiagonis* reaches a bigger size than *L. boscii* and the main distinctive morphological features are the presence of four black spots on *L. boscii*'s fins, absent in *L. whiffiagonis*, and some head differences. The dorsal fin of *L. boscii* originates close to the anterior edge of the eye while in *L. whiffiagonis* it is closer to the tip of the snout, the snout being sharper. The two species share most of their biological features and exhibit an overlapping distribution in the entire *L. boscii* habitat, which expands from the British Isles to Cape Bojador (26ºN) including the West Sahara coast and the Mediterranean Sea (Nielsen 1990). They are caught in mixed fisheries together with other species by drift or bottom trawl. Most European countries fish megrims and, according to FAO, Spain and the UK are the first in landings (~70% of total) followed by France and Ireland. The two megrims are managed together as a single species (ICES WGHMM 2008) and catch data are not consistent across databases, mainly due to lack of identification of the two species in the FAO database where the species' names were *L. whiffiagonis* and megrims nei (= *Lepidorhombus spp.*; FAO 2005) until 2000 and *L. boscii* appears as an independent species after 2000.

The main purpose of this work was to determine the accuracy of visual identification of the megrims *L. boscii* and *L. whiffiagonis* at landings and markets by comparing visual and genetic identification of species in order to evaluate the risk of underreporting either species.

2. MATERIALS AND METHODS

2.1 Samples analyzed

Spain was selected because it is the top country in megrim fisheries (38% of total landings in 2009, and >42% in the last decade; FAO landing statistics). We chose markets and landings from five different parts of Spain (Galicia, Asturias, Madrid, Catalonia and Valencia) to purchase products of both megrim species labelled with their species name. A total of 264 specimens from markets (15-20 packages from each market, with three specimens per package for a total of 88 packages), and 239 individuals obtained from landings (45-50 individuals from each port) were analyzed. A piece of tissue (approx. 3 g of muscle) was taken from each sample and stored in 100% ethanol until it was analysed. Samples were collected from 2005-2009 randomly within each market and landing point. Only information from FAO or ICES is available about the constancy through time of market patterns for these fishes (*i.e.* proportion of individuals caught). In 2000 a change in commercial fisheries management (reduction in minimum catch size) was implemented, thus increasing the potential catches of *L. boscii.* Within the years considered in this study, no policy changes in megrim catches were reported nor relevant change was noticed.

2.2 Species identification by DNA analysis

DNA extraction was carried out employing the resin Chelex following Estoup *et al.* (1996). We employed differences in length of the nuclear gene 5S rDNA for identification of both megrim species. The 5S rDNA is a conserved locus composed of a coding sequence, typically 120 base pairs (bp) long and highly conserved across species, and a non transcribed spacer (NTS) which can differ in length and sequence among closely related species (*e.g.* Pendas *et al.* 1995). We amplified by PCR a fragment of the 5S rDNA locus in a GeneAmp PCR System 9700 (Applied Biosystems) employing the primers 5S-A 5´-TACGCCCGATCTCGTCCGATC-3´ and 5S-B 5´-CA-

GGCTGGTATGGCCGTAAGC-3´ designed by Pendas *et al.* (1995), in a total volume of 20 µl containing 0.1 µl of GoTaq polymerase at 5U/µl (Promega), 4 µl of 5x Promega Buffer, 2 µl of 25 mM $MgCl_2$, 2 µl of a 2.5 mM dNTPs mixture, 1 µl of each primer at 20 µM, 2 µl of sample DNA and 7.9 µl of bidistilled water. PCR amplification conditions were: 5 min of initial denaturation at 95ºC, followed by 35 cycles of 20 s of denaturation at 95ºC, 20 s of annealing at 65ºC and 30 s of extension at 72ºC, and a final extension at 72ºC for 20 min. Products were run in 2.5% w/v agarose gels at 100 V stained with 2 µl ethidium bromide (10 mg/ml) for visualization. We estimated product size by comparison to a standard 100 bp DNA marker (Promega). For determining the exact fragment size, we analyzed four individuals of each species by capillary electrophoresis in a 3100 Genetic Analyzer (Applied Biosystems) with a 36 cm capillary and POP 4 polymer in the Unit of Genetic Analysis of the University of Oviedo (Spain). The chromatograms were visualized employing the GeneScan 3.7 Analysis Software (Applied Biosystems). Electrophoresis conditions were those given as default by the manufacturer.

3. RESULTS

3.1 Species-specificity of the markers assayed

Species-specificity of the marker was confirmed for the two megrim species. All the individuals belonging to a given species yielded the same genetic pattern. For *L. whiffiagonis* the 5S rDNA yielded a main fragment of 217 bp and a secondary one (fainter in the gel) of 472 bp (Garcia-Vazquez *et al.* 2006). For *L. boscii*, fragment sizes were 233 (main) and 330 (secondary and fainter) bp long.

3.2 Misidentification of European megrims at landing

From the Spanish landings analyzed, as many as 89.96% of megrims were labelled as *L. whiffiagonis* and the rest as *L. boscii*. Visual identification was therefore not accurate because the genetic marker employed revealed that only 48.95% were *L. whiffiagonis* and the other 51.05% were *L. boscii* (Table 1) resulting in 41.01% misidentification of megrim landings, that can be considered very high. *L. boscii* was therefore underreported at landings. Although the proportion of *L. boscii* estimated within megrim landings was different for FAO and ICES records (38.75% and 6.34% respectively), it is clear that the real proportion of *L. boscii* was higher than declared from landings, at least in Spain.

Our next step was to extrapolate the results obtained from Spanish landings to the FAO megrim catch statistics. There is no record from illegal or unregulated megrim fishing, although some unreported or mis-reported fishing has been documented in European waters (*e.g.* for Spain in ICES WGHMM Report 2008, and for the UK in MRAG: Review of IUU fishing and developing countries, 2005). The estimates of total landings (including unreported or mis-reported landings) and catches (landings + discards) are considered in the FAO (www.fao.org) and ICES databases used (ICES WGHMM Reports 2006, 2008). According to the FAO, 38.8% of megrims landed in the period 2005-2009 were

Table 1
Proportion of *Lepidorhombus whiffiagonis* and *L. boscii* individuals expected from FAO and ICES catch reports in 2001, reported from Spanish landings (239 individuals from five ports) and determined from genetic markers.

Species	Expected from FAO catch reports	Expected from ICES catch reports	Reported from landings	Genetically determined
L. whiffiagonis	61.25% 9	3.66%	89.96% 4	8.95%
L. boscii	38.75% 6	.34% 1	0.04%	51.05%

L. boscii (32,575 tons). If the actual proportion of *L. boscii* in landings was 51%, the real number of catch tons for this species would be 41,817, underreporting 9,200 tons of *L. boscii*, almost 30% of the reported catch.

3.3 Incomplete labelling of megrims in commercial packages

European directives of application in Spain (laws (CE) NO 178/2002 and (CE) Nº 1759/2006) require that food and seafood labels indicate the complete scientific name of the species (*i.e.* genus and species, Latin binomial nomenclature) without inducing errors and in order to ensure a high level of protection for human health. Nevertheless a high level of inaccurate labelling was detected amongst the 88 commercial packages analyzed in this work. In three of the five locations considered no packages were correctly labelled. In most cases (63.5%) the commercial packages were ambiguously or incompletely labelled as "gallos" (the Spanish word for "megrim") or with any incorrect version of the generic name "*Lepidorhombus*" (*i.e.* Lepidorrombus, Lephidorhombus) without the species name and marketed as only one species. From the remaining 36.5%, more than 21.2% of the marketed packages (19 in total) were incorrectly labelled as the other species, and only 15.3% (13 packages) were correctly identified with the full scientific species name. In the erroneously labelled lots the direction of the misidentification was similar to that found in landings; seventeen packages were labelled as *L. whiffiagonis* and only two as *L. boscii* whereas they were genetically determined as nine *L. whiffiagonis* and ten *L. boscii* packages.

4. DISCUSSION

In this study we have found high levels of misidentification in megrims at different points of the commercial chain: both at landings and markets. In this case, as in some hake species, intentional fraud could be discarded because although the two species are morphologically very similar their trade price is the same. Identification errors of megrim species are easy due to their high morphological similarity; however each species has its own morphological characteristics that are enough to clearly distinguish them from each other (see Introduction).

Although incorrect identification would be expected to be similar in the two possible directions (labeling *L. whiffiagonis* as *L. boscii* and vice versa), we instead found systematic underreporting of *L. boscii*. There is no clear explanation for this misreporting direction; perhaps *L. whiffiagonis* are expected to be more abundant in the catch, as they supposedly were for decades, and new catches rapidly labelled on board tend to be given that name. Or, perhaps progressively smaller adults (with less marked distinctive spots and easily confused with *L. whiffiagonis*) are being caught as bigger individuals are removed from the stocks by the fishery (*e.g.* Zhou *et al.* 2010). Regardless of the cause of mislabelling, underreported species are prone to overexploitation, which in the long term may lead to stock decline, exhaustion and even an eventual extinction of the overexploited species (Agnew *et al.* 2009). For example, the European common skate had been traditionally managed as one single species but is actually a composite of two different genetic units, the blue (*D. cf. flossada*) and the flapper skate (*D. cf. intermedia*); identification of the two species could save the flapper skate from extinction (Iglésias *et al.* 2010). In the case studied here, as in other cases (Marko *et al.* 2011), underreporting of *L. boscii* may endanger the conservation and sustainability of megrim fisheries since quotas depend on the number of individuals harvested from each species (Jacquet and Pauly 2008).

Other possible caveats about the origin of the mislabelling could be the potential economic gains to the individual fisher or processor. The possible motivation behind such behaviour could be "cost saving" methodologies. At landings we observed that fishermen recognized the two megrim species. However, as both species have the same market price, if they are sold as one the cost of separation is avoided at some point in the commercial chain. Thus giving a generic name (*i.e.* treat the two megrims as only one species) saves time and money.

The unidirectional mislabelling we detected causes underreporting of *L. boscii* and hence overreporting of *L. whiffiagonis*. Underreporting may lead to overexploitation and threaten the future of fisheries (Mora *et al.* 2009). This is unlikely to occur soon (although stocks conditions are unknown), but it may occur in the near future if mislabelling is maintained. The sustainability of fisheries is based on balancing catch and population size (Myers *et al.* 1995; Rose *et al.* 2001). Sufficient individuals have to be left for natural reproduction for maintaining a stable population without reduction in size. The amount of megrims allowed to be fished (TAC) is determined by their landings, therefore a correct identification of individuals at landings is necessary in order to know the status of their stocks to prevent unnoticed overexploitation, as in Marko *et al.* (2011) for Patagonian toothfish.

The implications of the mislabelling found here for the future of megrim fisheries remain uncertain; however these results should be taken into account for the establishment of the new TAC recommendations. In addition, there is a need for independent estimation of wild population sizes of the two species in order to hypothesize how they will change. Other examples of underreported species

(Agnew *et al.*, 2009; Iglésias *et al.* 2010; Marko *et al.* 2011) already show how bad a possible outcome could be. Effects of mislabeling for megrim fisheries sustainability should be acknowledged in future research, evaluating their population sizes, effective numbers and other relevant biological characteristics to resolve this issue.

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Biological Invasions

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Aquaculture as a gate for invasions. Hybrid zones of invasive NIS and native *Mytilus* mussels are linked with farms in Vancouver Island

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Abstract

Aquaculture promotes the introduction of non-indigenous species (NIS) into wild marine environments, especially when containment is not secured. NIS escapees may hybridize with natives and cause profound changes in their gene pool. Here we have sampled mussels from fourteen coastal locations with different farm densities in Vancouver Island (British Columbia, Canada). Two NIS, *Mytilus edulis* and *M. galloprovincialis*, are cultivated in those farms. Mussels were genotyped at two loci (Glu-5' and Me 15/16) with species-specific variants. The mitochondrial cytochrome c oxydase subunit 1 (COI) gene was sequenced to determine the maternal species in hybrids. The proportion of NIS and hybrids of *M. galloprovincialis* and the native *M. trossulus* was positively correlated with farm density. In addition, hybrids between the two native *M. trossulus* and *M. californianus* were also identified. Marine currents and different habitat preference of NIS provide additional explanations of the present distribution of alien and native species along Vancouver Island coasts. As a whole, our results emphasize the role of aquaculture as a vector for the introduction of invasive species and a promoter of hybrid zones.

Keywords: Aquaculture; Non-indigenous species; Hybrid zones; Introgression; *Mytilus*; Mussel farming; Genetic markers.

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1. INTRODUCTION

The introduction of non-indigenous species (NIS), for example, in farming, aquaculture, pest control, stocking for hunting/fishing, and even as pets, encompasses the risk of some escaping and becoming invasive, and is considered one of the most important environmental issues today (*e.g.* Grosholz 2002; Elliot *et al.* 2008). In aquatic habitats most NIS are crustaceans and molluscs and their introduction in a region is strongly associated with trade patterns, as a consequence of ship fouling (*e.g.* Ruiz *et al.* 2000). In addition, aquaculture is an increasing source of NIS (Naylor *et al.* 2000). Although aquaculture operations are supposed to be placed in isolated areas, and attempt to securely contain the farmed individuals, it is almost impossible to prevent escapes of gametes, larvae and/or adults (Heath *et al.* 1995). Commercial fish and shell-fish farming has thus led to the intentional and/or accidental introduction of alien aquatic species in many parts of the world (Hindar *et al.* 1991; Carlton 1992; Carriker 1992; Consuegra *et al.* 2011).

Not all the aquaculture escapees become invasive because domestic individuals are not always able to adapt to wild habitats (*e.g.* Hindar *et al.* 1991; Fleming and Gross 1993). However, many are able to survive, reproduce, and even displace the native species (*e.g.* Hindar *et al.* 1991), by competition and/or by hybridizing and introducing part of their genome in them (a process known as introgression; Anderson 1949). To cite a couple examples, Bekkevold *et al.* (2006) reviewed evidences for genomic introgression of farmed domestic salmon within natural populations modifying the native genetic pools; and Castillo *et al.* (2008) described interspecific hybridization and introgression as a result of interaction between wild and domestic *Salmo salar* and *S. trutta*. The extent of genomic introgression will depend mainly on the degree of domestication of cultivated stocks (how different they are from wild populations), and the quality and abundance of native populations (Danancher and Garcia-Vazquez 2011). The trace of introgressions may last for a long time, modifying the genomic pool of natives and causing unpredictable effects in the long term (*e.g.* Fitzpatrick *et al.* 2009, Lamaze *et al.* 2012). Hybridization has been considered an invasion of the genome with potentially important consequences in evolution, biodiversity and conservation (Mallet 2005). If invasive species also invade other species' genomes via introgressive hybridization, they will impact on the native community at multiple levels, not only ecological but also evolutionary. Hybridization following introduction of non-indigenous species is therefore a serious risk for native species, especially where they are rare (Rhymer and Simberloff 1996).

Taking into account the present scenario of increasing aquaculture activities, it is crucial to understand the conditions that favour invader's expansion and introgression into native gene pools. This knowledge would help to adopt precautionary approaches when establishing new aquaculture operations, and to establish scientifically-informed mitigation measures for helping native species and populations to recover from invasions. Although it is not easy to discriminate among factors that enhance invasions in complex environments, some interrelated processes that mediate invasion processes have been identified: changes in donor and recipient regions, invasion windows and dispersal vectors (reviewed in Carlton 1996). NIS take advantage of anthropogenic and/or disturbed habitats (*e.g.* Suchanek 1981; Linde *et al.* 2008; Shield *et al.* 2008). On the other hand, invasions also depend on resource availability in the recipient habitat (Stachowicz *et al.* 2002). A closer examination of areas where the invaders are currently in expansion is necessary in order to identify those factors that promote or restrict the invader's adaptation in a region.

Many examples of introgression of aliens escaped from farms into native gene pools, like those cited above, have been provided from fish. There are also some examples of NIS shellfish introduced via farming that are producing changes in the host environment and modifying the native fauna composition (*e.g.* Couceiro *et al.* 2012). Dispersal of farm escapees can be favoured by different factors such as marine currents or fouling (Carlton 1992; Geller *et al.* 1994; Gilg and Hilbish 2003). One case of successful shellfish NIS expanding in the new environment occurs on the North American Pacific coast and has the mussels of the genus *Mytilus* as protagonists.

Mytilus is one of the most thoroughly studied genera of marine molluscs (*e.g.* Kenchington *et al.* 1995). These mussels are important components of the intertidal community in rocky shores (*e.g.* Suchanek 1985; Koehn 1991). Within this genus, the *Mytilus complex* contains three species (*Mytilus trossulus*, *M. galloprovincialis* and *M. edulis*), which are morphologically quite similar and often difficult to identify visually (Heath *et al.* 1995). They are however genetically different and can be distinguished employing molecular markers such as allozymes (McDonald *et al.* 1991) or the 18S nuclear rDNA subunit (Kenchington *et al.* 1995). Some species are commercially important and are cultured worldwide, for example *Mytilus galloprovincialis*, which is farmed in Asia, America, Europe and Africa (www.fao.org). In the Pacific coast of North America, the native species are *Mytilus trossulus* and *M. californianus* (Morris *et al.* 1980; McDonald *et al.* 1991; Santaclara *et al.* 2006). Phylogenetically, *M. californianus* is the most divergent of the four species (Kenchington *et al.* 1995; Distel 2000; Martinez-Lage *et al.* 2002). The non-indigenous species (NIS) *M. edulis* (L) and *M. galloprovincialis* (L), both native to Europe (Quesada 1998; Hilbish 2000; www.fao.org), have been intensely farmed on North American Pacific coasts since the beginning of the 20th century (Hilbish *et al.* 2010). They have established feral populations there (Heath *et al.* 1995; Rawson and Hilbish 1995; Santaclara *et al.* 2006; Shields *et al.* 2010). *Mytilus galloprovincialis* is expanding its distribution and has been declared an invasive species (*e.g.* Braby and Somero 2006). In southern California it has even replaced the native *M. trossulus* (Geller 1999).

Hybridization among mussels in the *Mytilus complex* is frequent due to their related ancestry and shared habitat (Gosling 1992). Sympatric species often exhibit hybridization in this complex (Toro *et al.* 2004). The hybrids are fertile and able to produce backcrosses

(Hilbish *et al.* 2002; Beaumont *et al.* 1993; Wood *et al.* 2003). Natural hybrid zones are well known in Atlantic European (*e.g.* Bierne *et al.* 2002, 2003) and American waters (*e.g.* Comesaña *et al.* 1999). Hybridization between species of this complex has been described on the North American Pacific coast (*e.g.* Heath *et al.* 1995; Saavedra *et al.* 1996; Suchaneck *et al.* 1997; Rawson *et al.* 1999), as well as hybrid zones involving native and cultured species (Shields *et al.* 2010). However, hybridization between the two native *M. trossulus* and *M. californianus* has not been reported to date.

Given the known hybridization and introgression between *Mytilus* species, and the current expansive trend of *M. galloprovincialis* in the North American Pacific coast, this zone seems to be a good case study for investigating environmental factors that may promote ecological and evolutionary aquaculture-derived invasions. The present study is focused on the Vancouver Island (British Columbia, Canada; Figure 1), where mussel farms are concentrated in some locations whereas other zones are almost free of mussel aquaculture (www.dfo-mpo.gc.ca/index-eng.htm). Mussels were sampled from rocky shores in areas that varied in exposure to open sea, waves, type of substrate, algae coverage, farm density and also other anthropogenic factors such as distance to ports and substrate modifications. We used molecular markers to identify species and to determine pure or hybrid status. Our departure hypothesis was that areas of high farming density and concurrence of other anthropogenic disturbances will exhibit higher levels of interspecific introgression and hybridization between native and NIS mussels than less disturbed areas with similar farming density. In addition, currents may favour expansion of hybrid zones to less intensely farmed areas.

2. MATERIAL AND METHODS

2.1 Ecological and geographical setting of the study area

Mussels of the genus *Mytilus* are highly fecund spawners and their larvae can survive in the water for up to 2 months (Suchanek 1985; Bayne 1965). They exhibit a high dispersal capacity (range estimated between 30 to 60 Km, but >100 Km in some cases; McQuaid and Phillips 2000; Gilg and Hilbish 2003). Accordingly, extensive dispersal and panmixia among coastal populations is expected for this genus (Shields *et al.* 2010). However, Vancouver Island may be an exception for the reasons explained below.

Vancouver Island (VI) is separated from the British Columbia mainland by the Strait of Georgia (Fig. 1), which is a long (220 Km), narrow (18-55 Km) and deep (156-420 m) fjord estuary (www.nwfsc.noaa.gov/publications/techmemos/tm44/environment.htm) with small islands, peninsulas and shallow areas which restrict water flow (Herlinveaux and Tully 1961; Masson and Cummins 2004). Although wide water gyres occur all year round, they are stronger in summer (Masson and Cummins 2004) coincident with the mussel spawning season and may act to restrict larval dispersal (*e.g.* Toro *et al.*, 2004). The northeast part of VI has an assemblage of small islands and peninsulas with very narrow passages that may also limit water flow

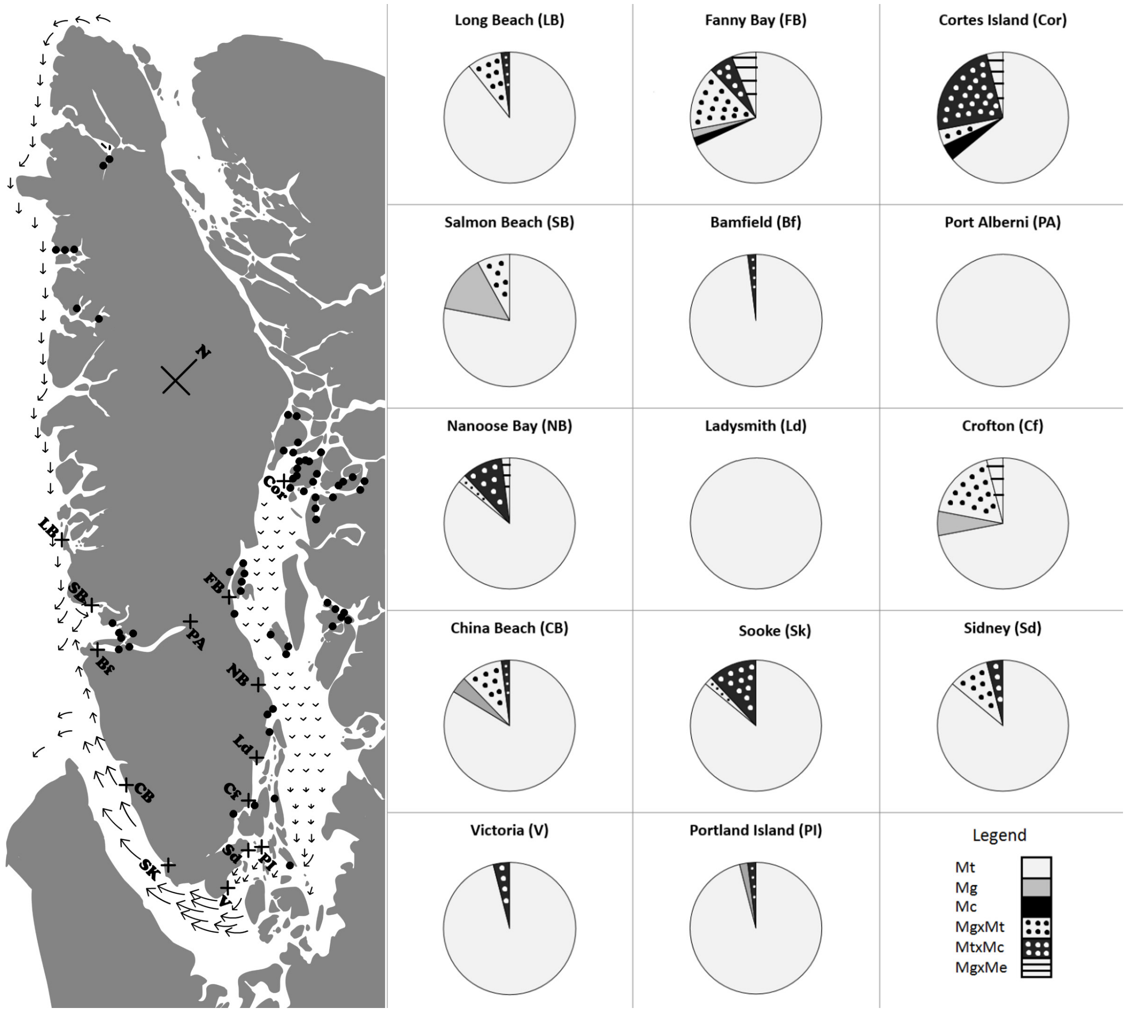

Fig. 1
Map of Vancouver Island including the sampling locations (crosses), aquaculture farms (dots) and prevailing marine surface currents (arrows; arrow sizes are indicative of current intensity). Also, percentages of genotypes found at each location are shown on the right side. Mt: *Mytilus trossulus*; Mg: *M. galloprovincialis;* Mc: *M. californianus;* MgxMt: *M. galloprovincialis x M. trossulus* hybrid; MtxMc: *M. trossulus x M. californianus* hybrid. Location names are given in Table 1.

and seems to isolate the northern region from the rest of VI (Heath *et al.* 1995; Shields et al. 2010). The West coast of VI is open to the Pacific Ocean and thus exposed to strong North-South marine currents near the shelf (las.pfeg.noaa.gov/oscurs; Fig. 1). Therefore, the convoluted hydrology and topology surrounding VI suggest intricate larval dispersal paths (Shields *et al.* 2010), resulting in localized population subdivision. The marine currents around the island depicted in Fig. 1 were obtained from the Department of Fisheries and Oceans Canada (www.dfo-mpo.gc.ca/index-eng.htm), the NOAA Ocean Surface Current

Simulator (OSCURS) (las.pfeg.noaa.gov/oscurs), and the Institute of Ocean Sciences of Canada (www.pac.dfo-mpo.gc.ca/science/facilities-installations/ios-ism/index-eng.htm) (Davenne and Masson 2001). The flood tide runs around the north and south ends of the island (from the Pacific side) and then meet at approximately the middle of the island on the east side. The species studied here are common in intertidal marine environments (Koehn 1991; McDonald *et al.* 1991). *Mytilus californianus* can be easily identified de visu (Fig. 2) because they exhibit strong radial ribs (Conrad 1837), and the other species are similar to each other, *M. trossulus* and *M. galloprovincialis* being almost identical (Linnaeus 1758).

Mussel farm locations and the species farmed were obtained from the records available at the Centre for Shellfish Research at Vancouver Island University (www.viu.ca/csr/industry/industrybackground.asp), the British Columbia Shellfish Grower's Association (http://bcsga.ca/about/industry-encyclopedia/mussels/) and the Department of Fisheries and Oceans of Canada (www.dfo-mpo.gc.ca/index-eng.htm; www.pac.dfo-mpo.gc.ca/aquaculture/licence-permis/docs/shell-conch-processors-transformateurs-eng.html). In addition to pure species (commonly *M. edulis*, *M. galloprovincialis* and a few farms with *M. trossulus*), British Columbia mussel farmers sometimes produce crosses *M. edulis* x *M. galloprovincialis* and triploids (www.dfo-

Fig. 2
Photographs of mussels sampled. *Mytilus trossulus* (top left), *M. galloprovincialis* (top right), and *M. californianus* (bottom).

mpo.gc.ca/index-eng.htm; http://bcsga.ca/about/industry-encyclopedia/mussels/).

2.2 Sample collection and preparation

A total of 700 adult *Mytilus sp.* individuals from 14 sites (Fig. 1; 50 samples per point) on Vancouver Island were collected randomly from rocky beaches of the intertidal zone (natural intertidal populations) between March and June of 2012. Sampling locations that varied with respect to mussel farm density, exposure to waves, algae coverage, substrate type (from natural to artificial such as stone or the most artificial concrete walls), distance to seaports, distance to freshwater and degree of sheltering from the open sea, were deliberately selected (Table 1). The sheltered sites experienced limited water flow and higher summer water temperatures, relative to the exposed sites (Heath *et al.* 1995). Sea ports with a harbour and regular ship traffic (ferry lines, commercial fish landing, big touristic ports) were considered. In addition, small boats travel all around the island and may stop at any shelter. These were not considered here.

At each location, samples were obtained from the intertidal transect, covering an area of 300-2000m2 depending on mussel abundance and habitat homogeneity. Shell measurements (length, width, and height) were obtained with callipers (±0.1mm). Valves were opened and the samples stored in 95% ethanol until genetic analyses were performed. The ethanol was changed twice on consecutive days to improve tissue preservation.

Table 1

Characteristics of the points sampled. Sampling site: (in order) Victoria, Bamfield, Sidney, China Beach, Sooke, Crofton, Ladysmith, Nanoose Bay, Fanny Bay, Salmon Beach, Long Beach, Port Alberni, Portland Island and Cortes Island; latitude and longitude; substrate type from 1 (natural) to 5 (totally artificial); distance to the nearest marine port (in Km); % algae coverage; wave exposure from 1 (none) to 5 (very exposed); number of farms in the surrounding coast (50 km); species farmed near the sampling location: *Mytilus galloprovincialis* as Mg, *M. edulis* as Me, *M. trossulus* as Mt; sheltering from marine currents (2, sheltered in a gulf or bay; 1, semi-exposed; 0, open waters); distance to freshwater (lake or river mouth, in kilometers); mean mussel shell measures, in mm.

Site	Lat	Long	Subs.	Dist Port	Algae	Wave exposure	Farms	Farmed species	Shelter	Freshwater	Length	Width	Height
V	48.411038	-123.299998	4	1.1	80%	4	0	0	0	0	23.33	12.77	10.54
Bf	48.830218	-125.136837	3	0	90%	3	7	Mg, Me	2	0	27.49	14.80	10.36
Sd	48.685879	-123.400997	2	0.34	30%	1	2	Mg, Me	0	0	22.48	12.57	10.50
CB	48.430226	-124.088539	2	30	10%	5	0	0	0	1	79.76	35.68	27.72
SK	48.365445	-123.714823	4	0	50%	1	0	0	0	0	32.96	18.13	12.80
Cf	48.855334	-123.616738	3	1.6	90%	1	5	Mg, Me	0	0	29.03	16.27	11.44
Ld	49.037256	-123.747480	3	6.8	90%	1	5	Mg, Me	2	0.9	32.98	18.47	13.73
NB	49.276779	-124.120329	4	2.3	10%	2	8	Mg, Me, Mt	0	1	28.00	15.16	11.35
FB	49.544327	-124.863123	4	2.9	60%	2	14	Mg, Me, Mt	0	0	33.83	17.81	13.97
SB	48.953473	-125.438088	2	9.3	50%	5	7	Mg, Me	0	0.53	46.24	25.28	20.61
LB	48.999087	-125.658151	1	18.7	50%	5	0	0	0	0.95	76.82	35.55	36.57
PA	49.170853	-124.829612	5	6.8	90%	1	0	0	2	1	33.34	16.02	12.35
PI	48.735007	-123.365201	1	4.2	20%	2	2	Mg, Me	2	0	33.79	17.13	14.10
Cor	50.105662	-125.053353	3	0.05	70%	3	21	Mg, Me, Mt	1	0.5	25.14	13.40	11.05

2.3 Genetic analyses

Total genomic DNA was extracted from a piece of 50mg (approx.) of mussel foot tissue, with 1mm silica beads and a PrepManTM Ultra sample preparation reagent (Applied Biosystems) following the manufacturer's instructions. DNA was stored at -20ºC.

Two different species-specific nuclear markers were employed for genetic identification of each sample. The locus Glu 5', which encodes a mussel polyphenolic adhesive protein, was PCR-amplified with the primers JH-5 and JH-54 (Rawson *et al.* 1996). The expected amplification products are one or two fragments 350/380 base pairs (bp) long for *M. edulis*, one 240 bp long fragment for *M. trossulus*, and one or two fragments of 300/500 bp for *M. galloprovincialis*. As a second species-specific marker, a part of the non-repetitive region of the foot protein 1 (locus Me) was amplified with the primers Me-15 and Me-16 (Inoue *et al.* 1995), which yielded amplification fragments 180 bp long for *M. edulis*, 168 bp long for *M. trossulus* and 126 bp long for *M. galloprovincialis*. The fragments obtained by PCR amplification of these two markers have not been reported for *M. californianus* previously and were determined in the present study.

PCR amplifications were carried out in a mixture of 17µl containing 0.5µl of DNA template, 7.88µl of distilled water, 4µl of buffer containing 7mM of $MgCl_2$ (Promega), 1.5µl of dNTP (2.5 mM, Invitrogen), 0.75µl of each pair of primers (0.2mM) and 0.12µl of Taq Polymerase (Promega, 5U/mL). The two markers were analyzed separately on an Eppendorf Mastercycler ep gradient S PCR machine. Amplification protocols consisted of an initial denaturation at 94ºC for 3min, 30 cycles of 94ºC for 20s, 48ºC for 20s and 72ºC for 45s, and a final extension at 72ºC for 15min for the locus Glu 5'. For the locus Me the protocol consisted of an initial denaturation at 95ºC for 5min, 30 cycles of 94ºC for 30s, 52ºC for 30s and 70ºC for 90s, and a final extension at 70ºC for 15min. A negative control, with no template DNA added, was included in all PCR assays. Amplified products were directly resolved in an 3% agarose gel stained with SYBR Safe DNA stain (Invitrogen) by comparison with a 50bp DNA ladder (New England BioLabs).

The cytochrome c oxidase subunit I (COI) was amplified with the primers COI-H and COI-L (Folmer *et al.* 1994) and the PCR conditions described therein to confirm species status for *M. californianus*. For species identification sequences were compared with the NCBI database, using a threshold of 98% identity. Since mussel mitochondrial DNA exhibits biparental inheritance, the type of sequence (female as F or male as M) informed on the cross direction in the hybrids found.

2.4 Statistical analysis

The environmental variation of the study sites was analyzed employing a principal component analysis (PCA) with the software PAST (Hammer *et al.* 2001), with Correlation option and 0.7 Jolliffe's cut. The contribution of the site characteristics to the total variance was estimated, as well as the correlation between sampling site characteristics and the proportion of pure species and hybrids. The genetic contribution of a species to the mussel gene pool of a population (sampling site) was the frequency of the corresponding species-specific Glu' 5 allele. Heterozygotes (hybrids) provide one allele and homozygotes (pure species) two alleles each. Besides the total number of farms we have also considered independently the presence/absence of each farmed species (*M. edulis*, *M. galloprovincialis*, *M. trossulus*) per site. We have used non-parametric statistics (Kendall's Tau correlation tests) given the non-parametric nature of many data (farm density, wave exposure and others). When correlation values >0.8 were obtained between two parameters, one of those was eliminated from the PCA to avoid auto-correlation biases.

In addition we have compared the abundance of NIS among categories of sampling sites (categorized by the number of farms per 50 km coast as low-, medium- and high-density) employing non-parametric contingency Chi-Square tests.

3. RESULTS

3.1 Identification of Mytilus californianus with the assayed markers

For the Glu 5' locus, a fragment 140 bp long was obtained by PCR amplification from three individuals classified de visu as *M. californianus* sampled from the Fanny Bay and Cortes Island locations. The banding pattern was clearly distinguishable from other species (Fig. 3). The species identification was confirmed for these individuals by the COI sequence, which was typical of *Mytilus californianus* with more than 98% identity with reference sequences included in GenBank (above the threshold 97-97.4% commonly accepted for species identification with this gene in Barcoding projects; *e.g.* Meyer and Paulay 2005).

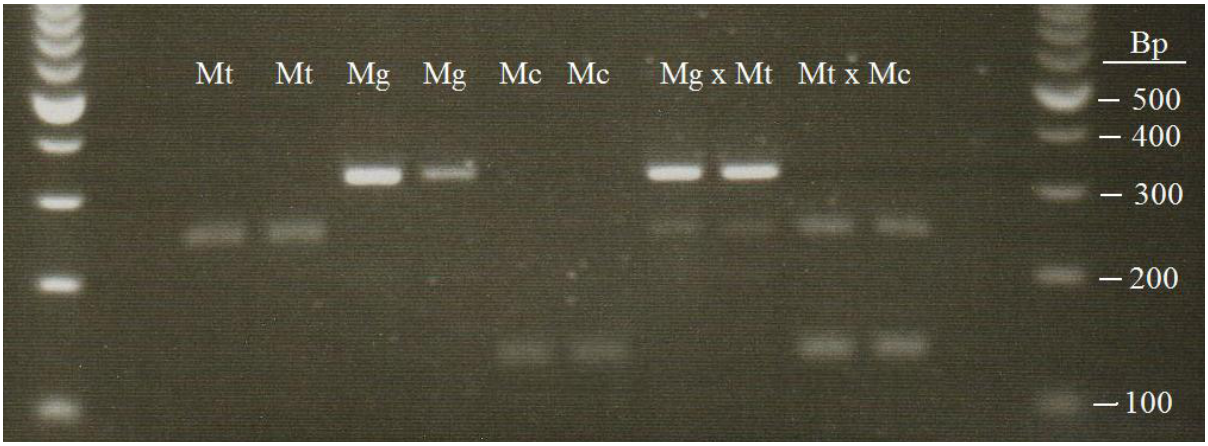

Fig. 3
Agarose gel (2%) stained with ethidium bromide containing PCR amplification products for the Glu-5' locus. Mt: *Mytilus trossulus*; Mg: *M. galloprovincialis*; Mc: *M. californianus*; MgxMt: *M. galloprovincialis x M. trossulus* hybrid; MtxMc: *M. trossulus x M. californianus* hybrid. A 100 bp ladder (Promega) was added as DNA fragment size marker.

The sequence obtained from these samples was uploaded into GenBank and is available under the accession number KC732796. Therefore a fragment 140 bp long for the Glu 5' locus can be considered typical for this species. On the other hand, the primers employed for the locus Me did not yield positive PCR amplification for the three *M. californianus* samples with the conditions assayed in this study. The Me locus was therefore used only for the other species in our survey.

3.2 Genetic identification of NIS and hybrids on Vancouver Island

Positive amplification of the Glu 5' fragment was obtained for 696 mussels (Table 2). Fragments of 380, 300, 240 and 140 nucleotides typical of *Mytilus edulis*, *M. galloprovincialis*, *M. trossulus* and *M. californianus* respectively, were retrieved from the samples analyzed. Individuals exhibiting a pattern with the typical fragments of two species simultaneously were classified as hybrids (Fig. 3). For the Me locus we found fragments of 180 (for one individual), 168 and 126 nucleotides typical of *M. edulis*, *M. trossulus* and *M. galloprovincialis* respectively, many of them simultaneously in the same individual (hybrid patterns) (Fig. 4). For *M. edulis* only hybrid patterns (with *M. galloprovincialis*) were found for the two loci. The genotype (pure species or hybrid) was not concordant for the two loci assayed in all the samples (Table 3). Many individuals exhibited a hybrid genotype for one locus and a pure-species genotype for the other, or even different pure-species genotypes (China Beach and Salmon Beach, see Table 3), indicating that they were post-F1 hybrids (F2 hybrids, backcrosses or further generations). From these results Vancouver Island can be considered a hybrid zone for the *Mytilus* genus.

Because the Me locus did not amplify for *M. californianus*, we only performed statistical analyses on the results obtained for the Glu 5' locus, *i.e.* only one locus, knowing that the hybrid status may correspond either to F1 or post-F1 hybrids. The most abundant species was the native *M. trossulus* (Table 2, Figure 1) with an overall percentage of 81.6%, followed by hybrids between *M. trossulus* and the in-

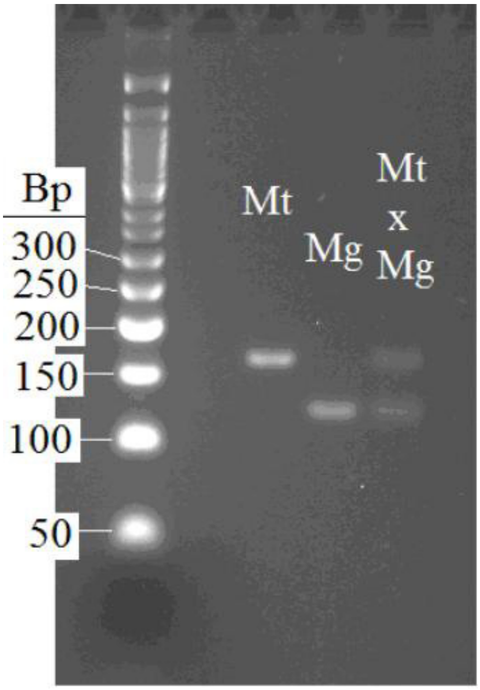

Fig. 4
Ethidium bromide-stained agarose gel (2%) showing PCR products for the Me locus. Mt: *Mytilus trossulus*; Mg: *M. galloprovincialis*; MgxMt: *M. galloprovincialis x M. trossulus* hybrid. In the first lane, 100 bp ladder (Promega).

Table 2

Observed percentages of Glu 5' alleles of *Mytilus trossulus* (Mt), *M. californianus* (Mc), *M. galloprovincialis* (Mg) and *M. edulis* (Me) in the sampling locations examined. Percent of NIS x native (FxN as Foreign and Native) and native x native (NxN) hybrids. Sampling site (in order): Victoria, Bamfield, Sidney, China Beach, Sooke, Crofton, Ladysmith, Nanoose Bay, Fanny Bay, Salmon Beach, Long Beach, Port Alberni, Portland Island and Cortes Island. Sample size=50 individuals except for LB and CB (47 and 49 respectively).

Site	Mt	Mc	Mg	Me	*FxN*	NxN
V	98	2	0	0	*0*	4
Bf	99	1	0	0	*0*	2
Sd	93	2	5	0	*10*	4
CB	89.8	1	9.2	0	*10.2*	2
SK	93	6	1	0	*2*	12
Cf	81	0	17	2	*18*	0
Ld	100	0	0	0	*0*	0
NB	92	5	2	1	*2*	10
FB	81	5	11	3	*16*	6
SB	82	0	18	0	*8*	0
LB	94.6	1.1	4.3	0	*8.51*	2.13
PA	100	0	0	0	*0*	0
PI	97	1	2	0	*0*	2
Cor	78	16	4	2	*4*	24

Table 3

Proportion of individuals of pure-species and hybrid genotype considering the two loci analysed (Me 15/16 and Glu-5') together. PP, pure species for the two loci; HH, hybrid for the two loci; PH, pure species for one locus and hybrid for the other; Discordant PP, one pure species for one locus and a different pure species for the other. Only individuals yielding clear genotypes for the two loci were considered.

	PP	HH	PH	Discordant PP
LB	0.93	0	0.07	0
CB	0.80	0	0.14	0.06
PA	1	0	0	0
V	0.96	0	0.04	0
Sd	0.86	0.02	0.12	0
PI	0.96	0.02	0.02	0
NB	0.86	0.04	0.10	0
SB	0.79	0.02	0.06	0.13
Bf	0.98	0	0.02	0
SK	0.86	0.02	0.12	0
Cf	0.72	0.12	0.16	0
Ld	1	0	0	0
FB	0.67	0.22	0.10	0
Cor	0.67	0.04	0.29	0

vasive *M. galloprovincialis* (5.6%, 39 individuals). Hybrids between the two native species *M. trossulus* and *M. californianus,* reported here for the first time based on Glu 5' amplification patterns, were also relatively abundant (4.9%). Pure *M. galloprovincialis* (2.1%), eight hybrids between the two NIS (*Mytilus edulis* x *M. galloprovincialis* individuals, 1.1%) and three pure *M. californianus* (0.4%) completed the total sample.

COI sequences were obtained from individuals with hybrid genotypes, as well as from individuals classified as pure alien species, in order to identify the maternal species and confirm their species status, respectively. The different haplotypes obtained were submitted to GenBank and are available under the accession numbers KC732781 to KC732805. As stated above, one of the individuals of phenotype and genotype *M. californianus* provided the *M. californianus* COI sequence, confirming its status. The individuals that yielded genotypes consistent with pure *M. galloprovincialis* for the two nuclear markers had COI sequences typical of *M. galloprovincialis*. The results obtained for the hybrids showed that *M. trossulus* x *M. galloprovincialis* individuals were produced from initial crosses with native *M. trossulus* mothers in 44.4% of the cases (consequently alien *M. galloprovincialis* females in 55.6% of the cases). For *M. trossulus* x *M. californianus* hybrids, *M. trossulus* was the mother in 88.2% of the cases.

3.3 Environmental conditions associated with NIS and hybrids

In the PCA four components accounted for more than 80% of the total variance (Table 4). The main environmental factors contributing to these components (marked in bold on Table 4) were distance to ports, wave exposure, degree of sheltering (from open waters), distance to freshwater, farm density and finally subtrate. Algae coverage contributed less to

Table 4
Results of the Principal Component Analysis for environmental factors of the sampling sites, indicating the variance explained by each component (in percent) and the relative effect of each factor within each component. The two highest values within each component are marked in bold.

	Component 1	**Component 2**	**Component 3**	**Component 4**
Variance	38.65%	18.23%	14.29%	13.10%
Substrate	-0.387	0.206	0.158	**0.700**
Distance to Port	**0.542**	0.281	0.007	0.067
Algae coverage	-0.391	0.350	0.124	-0.094
Wave exposure	**0.456**	-0.054	**0.371**	-0.157
Farm density	-0.211	-0.154	**0.854**	-0.230
Shelter	-0.229	**0.594**	-0.149	**-0.581**
Freshwater	0.314	**0.613**	0.245	0.281

the variation among sampling sites. The distance to ports was positively correlated with mussel size (ʈ = 0.633, *P* =0.001), as it could be expected (better mussel growth in cleaner sites). However, the distance to ports was not significantly correlated with the frequency of any species or hybrids.

Species composition was not homogeneous along VI coast and differences among sites were found in terms of abundance of the four species and their hybrids (Fig. 1, Table 2). Samples from four sites (Port Alberni, Victoria, Bamfield and Ladysmith) contained exclusively alleles of native mussels (*M. trossulus* and *M. californianus*). The other sites exhibited considerable differences in the proportion of NIS (pures and hybrids) (Table 2). NIS gene frequency was up to 0.19 in Crofton, being 0.18 and 0.16 in Salmon Bay and Fanny Bay respectively. The frequency of *M. galloprovincialis* genes was negatively associated with shelters (ʈ = -0.546, *P* = 0.006). *M. edulis* was significantly associated with farm density (ʈ = 0.608, *P*= 0.002).

For the natives, the frequency of *M. trossulus* alleles was positively associated with shelters (ʈ = 0.442, *P* = 0.027) and negatively correlated (as expected) with NIS gene frequency (ʈ = -0.642, *P* = 0.001). Besides, farming of *M. trossulus* was strongly positively correlated with hybridization between natives (generally female *M. trossulus* x male *M. californianus* as explained above): ʈ =0.554 with *P*= 0.005. This strongly suggests that farmed *M. trossulus* hybridizes with the other native species, *M. californianus*.

The effect of farms on NIS abundance was not straightforward since some sites with relatively quite dense farming (Nanoose Bay and Bamfield with 8 and 7 farms respectively) contained a few or no NIS genes. It was however evidenced when sampling sites were grouped by farm density. Sites located in areas with relative high density of farms (at least 5 per 50 km coast) contained globally 13.1% individuals with NIS alleles, considering both pure NIS and hybrids NIS x Native, compared with only 5.2% (also globally) in zones with less than 5 farms per 50 km coast. The difference in abundance of individuals with NIS alleles between areas of different farm density (pooling the sites with <5 farms/50 km in a group and the rest of farms in another) was highly significant (Chi-Square of 13.28, 1 degree of freedom, *P*<0.001).

4. DISCUSSION

The results presented in this study reveal a significant association between mussel farms and hybrid zones. Two types of hybrid zones were detected: one involves the two native species and is statistically correlated with farming of one of the natives, *M. trossulus*; the other involves native *M. trossulus* and NIS *M. galloprovincialis*. Although the hybrid generation of native x native hybrids cannot be determined with our data (the Me marker did not yield positive amplification from *M. californianus* DNA), the NIS x native post-F1 hybrids found in this study support the view of Vancouver Island as a hybrid zone for mussels (Shields *et al.* 2008, 2010). Two decades ago, Heat *et al.* (1995) already reported hybridization between native *Mytilus trossulus* and introduced *M. galloprovincialis* and *M. edulis*. However, *M. edulis* was found in in very low frequency in the present study, and only hybridized with the other NIS. It is possible that the two European species compete there, as reported in native areas (Hilbish *et al.* 2002), and that the more invasive *M. galloprovincialis* (Braby and Somero 2006) is out-competing *M. edulis* on the island. Another indicator of *M. galloprovincialis*' expansive trend is the fact that it provides the female in most hybrid crosses, a typical behaviour of pioneers and colonizers (*e.g.* Horreo *et al.* 2011 and references therein). Moreover, it seems that the level of introgression is increasing in Vancouver Island. Heath *et al.* (1995) reported 6.2% aliens, whereas Shields *et al.*'s (2010) figures were 4.2% and 6.6% in 2005 and 2006 respectively. The value of 13.1% found in our work for the 2012 samples obtained from farming areas (5.2% in areas with little farming), although not strictly comparable with previous studies due to a different sampling strategy, suggest that farms are acting as vectors of exotic mussels in Canada and also confirm the invasive condition of *M. galloprovincialis* (Geller 1999; Braby and Somero 2006).

For the other type of hybrids found in this study, *M. trossulus* x *M. californianus*, its frequency was associated with M. trossulus farms, suggesting that hybrid crosses involve domestic (farmed) individuals of this species. The maternal species was *M. trossulus* in most cases. This result reinforces the idea of hybrids promoted by farming since domestic stocks are expected to provide the female in hybrid crosses, as demonstrated in escapes of farmed Salmonids (*e.g.* Matthews *et al.* 2000) and other fish (*e.g.* Cross 2000). It is the first time that hybridization between these two genetically different *Mytilus* species is evidenced, and this result strongly suggests high disturbance of natural reproduction of VI mussels –presumable due to farming.

The present study emphasizes the role of aquaculture as a vector of spreading species and its role in maintaining hybrid and introgressed genotypes. Differences in habitat preferences among species have been reported for the *Mytilus complex* (Gilg and Hilbish 2003; Shields *et al.* 2010). Interestingly, hybrids and pure species showed different habitat preferences. Although *M. trossulus* is positively correlated with shelters in our study, the hybrids *M. trossulus* x *M. californianus* are not, which is consistent with the fact that *M. californianus* has an advantage in exposed habitats (Bell and Gosline 1997). Moreover, hybrids between native *M. trossulus* and invasive *M. galloprovincialis* seem to prefer exposed zones (they are negatively associated with sheltering). The plasticity of invader life history traits is important for promoting invasions (*e.g.* Facon *et al.* 2008; Valiente *et al.* 2010). Hybrid zones could be thus seen as expansion zones where individuals with mixed genomes are able to colonize new habitats. Higher invasiveness capacity of hybrids in comparison with the parental species has been described in plants (*e.g.* Ayres *et al.* 2004; Prentis *et al.* 2008), and aquatic animals (*e.g.* Facon *et al.* 2005; Nolte *et al.* 2005). In the present case different habitat preference would represent a way for hybrids with invasive *M. galloprovincialis*' genomes to settle in exposed areas.

Although farming has been found here as a main factor for the spreading of exotic mussels on Vancouver Island there are other possible vectors. A well recognized vector for transferring exotic mollusc species is fouling or boat transport (Carlton 1992; Geller *et al.* 1994). However, no significant correlation of NIS with distance to ports was found in our study, supporting Heat *et al.* (1995) who suggested that fouling is not a major factor of mussel dispersal in the region. Another factor contributing to mussel dispersal are marine currents (Gilg and Hilbish, 2003; Shields *et al.* 2010). In this study there are some hints of their importance. For example, Sooke and China Bay have no farms nearby but contained some NIS. Both areas are affected by marine currents that may carry mussel larvae from more intensely farmed areas like Crofton and mainland British Columbia farms (Fig. 1).

The dynamics of mussel hybridization and introgression on Vancouver Island cannot be explained from known environmental gradients alone (Shields *et al.* 2008). Although there are no available data about the proportion of each species in each farm and the possible occurrence of farmed hybrids (this type of farm escapes could explain *M. edulis* x *M. galloprovincialis* hybrids found in our study), our results indicate that farms may help to understand the hybridization patterns, likely in combination with a mixture of selection and barriers to dispersal (Veliz *et al.* 2006). Dias *et al.* (2009) reported higher levels of hybridization between *M. trossulus* and *M. galloprovincialis* in Scottish aquaculture sites than in natural intertidal populations. Heath *et al.* (1995) reported a decrease in the incidence of alien mussel alleles with the distance to farms in southeastern Vancouver Island, similar to our results found near the intensely farmed areas of Cortes Island and Fanny Bay.

Another finding of this study was the use of the Glu-5’ marker, already used for studies of hybridization in mussels (Hilbish *et al.* 2002; Gilg and Hilbish 2003), for identification of Mytilus californianus and its hybrids (confirmed with COI sequences). Since the different species are not easily visually identifiable (Heath *et al.* 1995), even less their hybrids, this marker will serve for further studies on mussel population genetics and introgression.

In conclusion, our results suggest that the larvae escaped from aquaculture farms are the main source of domestic mussels that interact with the native species and form hybrid zones on the Pacific coast of Canada. Hybrids zones may be considered gates for invasions when hybrids acquire new habitat preferences. In combination with marine currents that may facilitate their dispersal, mussel farms seem to be creating such type of hybrid zones on Vancouver Island. The present study adds to the understanding of the effects of aquaculture escapes and their implications in invasion processes.

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Interspecific Hybridization Increased in Congeneric Flatfishes after the *Prestige* Oil Spill

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Abstract

Marine species with relatively low migratory capacity are threatened by habitat alterations derived from human activities. In November 2002 the tanker *Prestige* sank off the Spanish northwest coast releasing 70,000 tons of fuel and damaging biota in the area. Despite efforts to clean the damaged areas, fuel remnants have affected marine species over the last nine years. This study is focused on two flatfish, *Lepidorhombus boscii* (four-spotted megrim) and *L. whiffiagonis* (megrim), whose spawning areas are located at the edge of the continental platform. We have analyzed megrim samples from North Spanish and French waters obtained before and after the oil spill. Genotypes at the nuclear marker 5S rDNA indicate a significant increase in interspecific hybridization after the *Prestige* accident, likely due to forced spawning overlap. The mitochondrial D-Loop region was employed for determining the direction of hybrid crosses, which were most frequently *L. boscii* female x *L. whiffiagonis* male. Reduced ability of *L. boscii* females to select conspecific mates would explain such asymmetric hybridization. To our knowledge this is the first time that increased hybridization between fish species can be associated to an oil spill. These results illustrate the potential long-term effect of petrol wastes on wild fish species.

Keywords: Interspecific hybridization; hybrid detection; oil spill; *Lepidorhombus boscii*; *Lepidorhombus whiffiagonis*.

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1. INTRODUCTION

Many human activities endanger survival of marine species. For example, wild populations are threatened by high ship traffic [1], overfishing [2] and many others, such as oil spill accidents [3]-[5], whose long term consequences have not yet been evaluated. Oil spills cause severe damage to marine wildlife due to polycyclic aromatic hydrocarbons (PAHs) released from fuel remnants causing oxidative stress, tissue alterations and cell death among other injuries by increasing oxygen-derived free radicals [6], [7 and references therein].

The *Prestige* oil spill occurred off the Galician coast (Northwest of Spain; Fig. 1) on 13th of November 2002, and was classified as one of the worst ecological catastrophes of the century in Europe [8]. The *Prestige* tanker carried about 77,000 tons of fuel type M-100 (one of the most toxic petroleum derivatives),

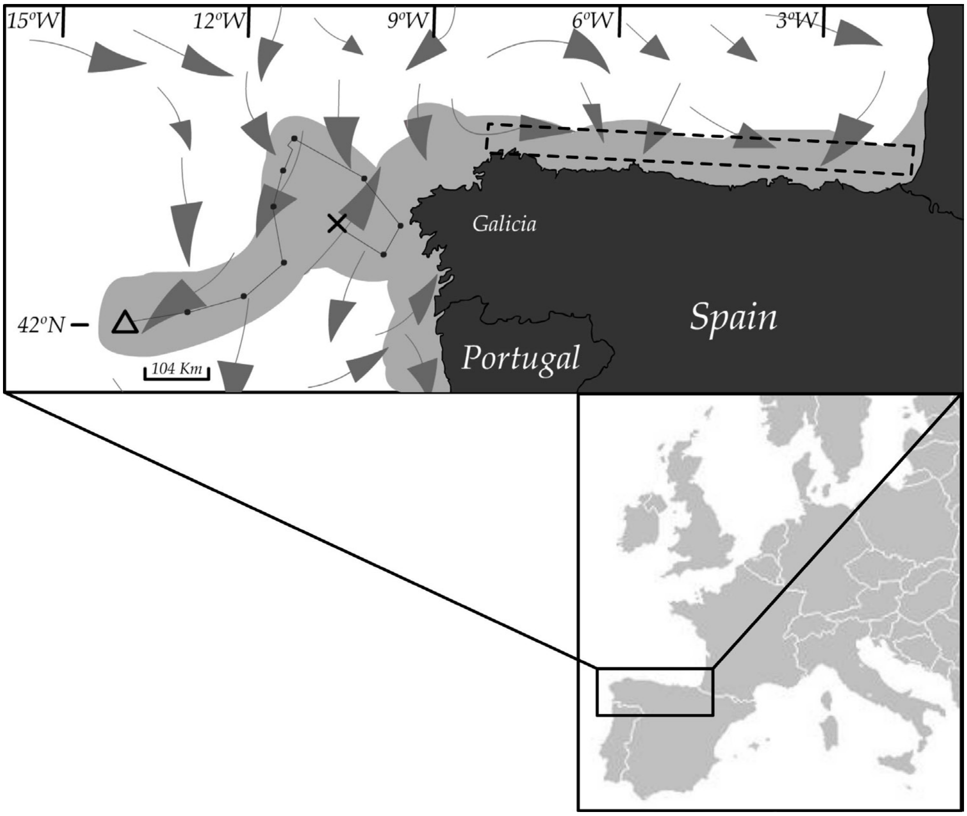

Figure 1
Map of the area affected by the *Prestige* oil spill and sampling area. Map of Northwest Iberia (Cantabric Sea) showing the location where the *Prestige* sunk (triangle off Galician coast) and its last trajectory (black line with dots) from the location of the accident and mayday alarm (cross). Arrows represent marine currents in the area and season of the accident (taken from http://oceancurrents.rsmas.miami.edu/). Their sizes are representative of their strength. The area affected by the oil spill is marked in light grey. The sampling area is marked as a dotted rectangle.

of which 20,000 tons were dumped directly into the sea at the time of the accident affecting an area of 30,000 km2 [9]. From then until the summer of 2003, 40,000 more tons were spilled [8] affecting marine communities of the Cantabric Sea and the Bay of Biscay along 2,600 coastal km [10], reaching the oyster farms in the Bay of Arcachon (France). Oil pollution caused a great damage to both marine biodiversity and the local economy [11]. Eight years after the disaster there were still at least 202 beaches contaminated by fuel in Galicia, and many others in Asturias, Cantabria and the Basque Country (www.wwf.panda.org).

Many marine vertebrates were affected by the oil spill including birds, mammals and fish [12], [13] and a review by [14]. Due to the high density of the fuel, it accumulated on the sandy bottom of the continental shelf. Species that swim and live on the bottom where the fuel accumulated were consequently most affected by the oil spill [8], [10], [15]. Previous studies carried out in the Mediterranean Sea demonstrated that the megrim *L. boscii* is a very sensitive species to PAHs exposure [16], and it was also especially affected by *Prestige* PAHs, which altered its DNA integrity and increased levels of stress and genotoxicity biomarkers [10].

Lepidorhombus boscii and its congeneric sympatric species *L. whiffiagonis* (Scophthalmidae, Pleuronectiformes) are distributed in the Atlantic Ocean from Iceland to Cape Bojador (26°N), and in the Mediterranean Sea. Both species spawn on the continental shelf from March to June. Little is known about the biology of megrims populations. Sanchez *et al.* [17] suggested low migratory capacity of both megrim species with aggregation and disaggregation movements. Surveys have shown that larvae do not move much from their spawning sites during the first year of life [18]. Spatial genetic differences have been described within their Atlantic area of distribution, megrims inhabiting the Bay of Biscay, Cantabric Sea, Galician and Portuguese coasts belonging to the same population cluster in the two species [19]. Studies on juveniles have been focused on development and growth patterns [17], [20], [21], but megrims' spawning areas have not been studied in depth and the reproductive barriers between the two species are unknown. The age at first maturity is 1.5 years for *L. boscii* [22] and two years could be reasonably considered their generation time; therefore approximately four generations (nine years) were affected since 2002 when the *Prestige* sank. The oil spill was quickly displaced during the 2002-2003 winter and in to a lesser extent until spring and summer 2003 [8] by both surface and deepwater currents, and fuel was deposited on the seabed, most likely reducing suitable megrim spawning areas and thus increasing reproductive interactions between the two species. Furthermore, the seabed was not cleaned up and the oil deposited likely has continued to affect megrim spawning since the spill until the present day. Interspecific hybridization can follow habitat alterations [23]. Our hypothesis is that the habitat alteration due to the *Prestige* accident has forced the two megrim species towards a closer interaction due to the reduction of "clean" spawning areas, especially *L. boscii* because of its higher sensitivity to fuel toxicity [10] and may be altering their mating behaviour. As a consequence of these factors, together or separately, we would expect increased interspecific hybridization between the two megrim species.

The aim of this study was to test whether interspecific hybridization between the two megrim species increased after the *Prestige* oil spill in the Cantabric Sea, which contained the coastal regions most affected by the catastrophe [9], [10]. The nuclear 5S rDNA locus, frequently used as species-specific marker in fish [24], and RFLPs (Restriction Fragment Length Polymorphisms) at the mitochondrial D-loop sequence for determining the maternal species of hybrids were employed as molecular markers for identifying interspecific hybrids.

2. RESULTS

Amplification of the 5S rDNA locus yielded different fragments for each species. *Lepidorhombus boscii* exhibits one main fragment 233 bp long and a secondary fragment of 330 bp (much weaker in the gels). For *L. whiffiagonis* we obtained two fragments of 217 (main) and 472 (secondary, also weaker) bp long, as described in [25] for Atlantic megrim. Before the Prestige accident, only one individual (0.75%) from the sampled area exhibited a pattern of amplification fragments which corresponded to an interspecific hybrid, containing the two main fragments of each species: 217 and 233 bp long (Fig. 2). The weaker species-specific secondary fragments also appeared but are less clear in agarose gels and were not considered in this study. The hybrid specimen had been classified de visu as *L. whiffiagonis*. After the accident the situation changed drastically (Table 1). A total of 38 individuals (25.67%) exhibited hybrid genotypes. Three of them exhibited a typical *L. whiffiagonis* phenotype and 35 were *L. boscii*-like. A Chi-Square analysis confirmed that the proportion of interspecific hybrids increased significantly after the *Prestige* accident (χ2= 36.54, 1 degree of freedom, *P*<<0.001). The species composition of the samples was also different, with more *L. whi-*

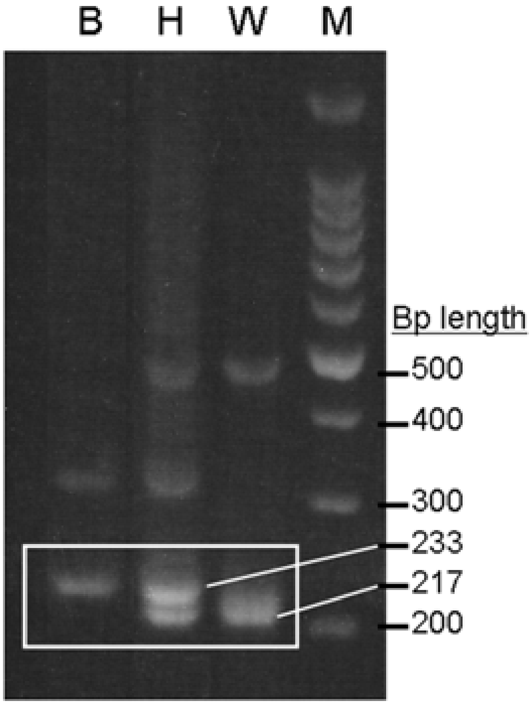

Figure 2
Agarose gel with amplification fragments of the 5S rDNA for pure and hybrid individuals. Agarose gel (2.5%) stained with ethidium bromide containing PCR amplification products of the 5S rDNA of *Lepidorhombus boscii* (B), the interspecific hybrid (H) and *L. whiffiagonis* (W). M: 100 bp ladder as DNA fragment size marker.

Table 1
Species identification of megrim samples obtained before and after the *Prestige* accident. Results are presented as the number (percent) of individuals. A first classification of individuals in interspecific hybrid or in each of the pure *Lepidorhombus* species was based on the 5S rDNA marker. Afterwards, hybrid individuals were classified as belonging to one of the two main species based on external phenotype and mitochondrial D-loop sequences (maternal species) as Hyb Lw (both *L. whiffiagonis* phenotype and mother) or Hyb Lb (both *L. boscii* phenotype and mother). N: total number of samples.

Trend	Organism	Taxonomic level	Bioindicator	Zone	Reference
Recovery	Mussels	Mollusks	Tissue-level biomarkers	Galicia, Basque Country	Cajaraville *et al.* (2006)
Recovery	Megrim Shrimp	Fish Crustaceans	Stock abundance	Basque Country	Sanchez *et al.* (2006)
Recovery	Mussels	Mollusks	PAH content	Galicia	Soriano *et al.* (2006)
Persistence	Polychaetes	Worms	PAH content	Galicia	Morales-Caselles *et al.* (2008)
			Composition of		

ffiagonis in the after-*Prestige* sample and more *L. boscii* in the before-*Prestige* one.

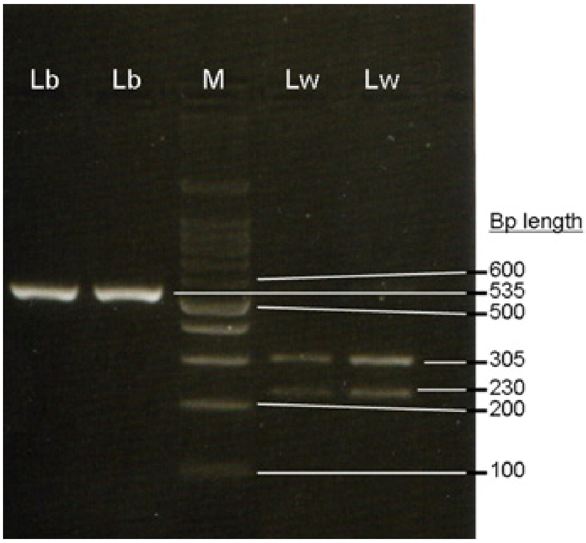

Figure 3
Agarose gel (2.5%) showing the different Dra I patterns for megrims D-loop. Lb: *Lepidorhombus boscii,* M: 100 bp DNA ladder, Lw: *L. whiffiagonis.*

The direction of hybrid crosses was assessed from D-loop RFLP. Amplification of this region with D-loopDF and D-loopDR primers yielded one fragment 535 bp long for both *Lepidorhombus* species. After digestion with Dra I, individuals with *L. boscii* mitochondrial D-loop (without restriction targets for this enzyme) yielded the same uncut fragment of 535 bp, as expected. Individuals with *L. whiffiagonis* mitochondrial DNA, with one Dra I restriction target, provided two fragments 230 and 305 bp long (Fig. 3) as also expected from the sequences. The 35 hybrids classified de visu as *L. boscii* exhibited a L. boscii D-loop pattern, and the three hybrids morphologically identified as *L. whiffiagonis* possessed a *L. whiffiagonis* D-loop (Table 1). As mitochondrial DNA is maternally inherited, we can conclude that most of the hybrid crosses that occurred after the *Prestige* accident (92.1% of the hybrids found in this survey) corresponded to *L. boscii* females mating with *L. whiffiagonis* males. All the hybrids were classified de visu as belonging to the maternal species, indicating that the external phenotype could be heavily influenced by the mother in interspecific crosses.

3. DISCUSSION

As the first long-term investigation of the area, the results found in this study reveal an increase in hybridization between the two megrim species in an area especially affected by the *Prestige* oil spill [9], [10]. The proportion of hybrids changed from less than 1% to more than 25% in only nine years and, according to the D-loop region, most hybrid crosses involved *L. boscii* females and *L. whiffiagonis* males. Increased hybridization between sympatric species following environmental disturbances has been observed in a wide range of plant and animal taxa [26]. For example, the proportion of interspecific hybrids between stickleback species from Enos Lake (Victoria, British Columbia, Canada) increased from 1% [27] to 12% [28] or 24% [29] following an anthropogenic-derived ecological change (introduction of an exotic predator) [30]. A species breakdown has been suggested for sticklebacks [29], [30], [31]; however this not seems to be the case for megrims, in which only part of one area/population of the whole distribution was affected, similar to a unique event of hybridization increase. The appearance of many hybrids in the studied area may not be directly attributed to habitat loss caused by the petrol waste because interspecific matings on affected sea bottoms have not been physically observed. However, it seems to be at least one of the causative factors because loss or alterations of habitats are frequently implicated as contributing factors in hybridization in fishes [32]. In other species such as cichlids, loss of water transparency has caused the rupture of pre-mating barriers based on body coloration, increasing the proportion of hybrids up to 88% [23].

Interspecific megrim hybrids may have accumulated in the studied region after the environmental degradation during the four generations elapsed since the accident. In addition to reduced and deteriorated habitat, and although interspecific mating barriers have not been studied for megrims, stress conditions in the area could have affected the mating behaviour of megrims, which is the second part of our hypothesis. In some amphibians stress affects the quality of male vocalization which is a determinant of female choice [33], [34]. Altered behaviour of females, particularly the rejection of allospecific males, may exlain many cases of unidirectional hybridization [35]. For example, rodents of both sexes prefer conspecific over congeneric individuals in normal non-stress conditions, but mate choice may change if the hormonal balance is altered, as happens in stressful conditions [36]. Other studies show that males of high body condition are often preferred by females due to the relationship between body size and male quality or fitness [37], [38]. This could explain the higher proportion of hybrids with a *L. whiffiagonis* father and *L. boscii* mother as *L. whiffiagonis* are larger than *L. boscii*. Although we don't understand the particular underlying mechanism, it is possible that *L. boscii* females were more receptive to accepting males of the other species due to hormonal changes (probably pheromones) in response to the ecological stress caused by the spillage and that those pheromones could act as a pre-mating barrier in non-stressful conditions.

An alternative explanation is that hybrid crosses have not increased but instead the fitness of hybrids has been enhanced. Changes in the fitness of hybrids before and after environmental perturbations have been reported in the scientific literature for both plants and animals [38], [39]. In fish, as in other species, hybrids may constitute a mechanism by which species deal with marginal habitats and environmental deterioration [40] and may even enable colonization of new habitats [41], [42]. Whether hybrid crosses increased directly by forced spawning overlap of the two species when changes in hormones relaxed mating choice, and/or hybrid fitness has increased due to environmental deterioration, the final result was an increase in interspecific hybridization in the area affected by the petroleum.

The difference between pre- and post-*Prestige* samples in the proportion of pure individuals of each species could be explained by differential sensitivity of the two species. Martínez-Gómez *et al.* [10] showed that *L. boscii* was strongly sensitive to the *Prestige* toxic wastes, and the decrease of this species in post-*Prestige* megrim samples is consistent with such high sensitivity, indicating a possible decline in its population size. In addition, the hybridization that occurred after the oil spill was asymmetrical, with a higher proportion of hybrids resulting from *L. boscii* females. The rare species frequently provides the female in hybrid crosses [36], [43], consistent with what we observed in our study with *L. boscii*.

In conclusion, a high increase in interspecific megrim hybrids in the northern Spanish area affected by the *Prestige* oil spill may suggest that the accident could have increased the interspecific hybridization rate between megrims, likely due to a combination of altered mating behaviour and reduction of suitable spawning habitat. These hypotheses should be verified with future work and, if proven correct, the consequences of such interspecific introgression should be examined in further surveys. Although the trace of alien introduced genomes will likely remain for generations, measures for helping the most affected species *L. boscii* including a reduction in fishing mortality by increasing the allowable megrim size at catch, should be considered for future conservation of these valuable flatfish.

4. MATERIALS AND METHODS

4.1 Samples analyzed

One year before the *Prestige* accident (August 2001) muscle fragments from adults of the two *Lepidorhombus* species were collected during research cruises and identified de visu by technical staff of the Spanish research institutions AZTI (a technological centre specialised in marine and food research) and IEO (Spanish Institute of Oceanography) from the Cantabric Sea and Bay of Biscay (corresponding with the ICES area VIIIc). Species classification was made according to head differences between species and the four characteristic spots typical of *L. boscii*'s fins (see Fig. 4). *L. whiffiagonis* individuals present a sharp snout, which is also approximately two-times bigger than their eye diameter, and the dorsal fin origins closer to the tip of the snout than to the anterior edge of the eye. Otherwise, *L.*

Figure 4
Pictures of megrim species. Pictues of body (up) and head (down) of *L. whiffiagonis* (left) and *Lepidorhombus boscii* (right).

boscii individuals' dorsal fin originates closer to the anterior edge of eye and presents a smaller snout length than *L. whiffiagonis*. In total 39 *L. whiffiagonis* and 95 *L. boscii* were sampled.

In July 2011, nine years after the oil spill, new adult samples (heads or whole individuals) of the two megrim species were collected randomly from fishing vessels operating across Cantabric Sea and Bay of Biscay waters (similar locations as in 2001). In total we sampled 101 *L. whiffiagonis* and 47 *L. boscii*. They were visually classified as explained above. Only two phenotypic morphs were found across all individuals (corresponding with *L. boscii* and *L. whiffiagonis* "typical" individuals) and no morphological differences were found among individuals belonging to the same species.

A piece of gill or muscle tissue (approx. 3 g) was taken from each sample and stored in 100% ethanol for genetic analyses.

4.2 Genetic analyses

Total genomic DNA was extracted following a Chelex based protocol [44] and stored at 4ºC. We amplified the 5S rDNA locus employing the primers A 5’-TACGCCCGATCTCGTCCGATC-3’ and B 5’-CAGGCTGGTATGGCCGTAAGC-3’ designed by [24] in 20 µl of total volume containing 4 µl of 5x Promega Green Buffer, 2 µl of 25 mM $MgCl_2$, 2 µl of a 2.5 mM dNTPs mixture, 1 µl of each primer at 20 µM, 0.1 µl of GoTaq polymerase at 5U/µl (Promega), 2 µl of sample DNA and 7.9 µl of bidistilled water. PCR amplification cycles were: 5 min of initial denaturing at 95ºC, followed by 35 cycles of denaturing at 95ºC for 20 s, annealing at 65ºC for 20 s and extension at 72ºC for 30 s, plus a final extension at 72ºC for 20 min. Amplification products were run in 2.5% w/v agarose gels at 100 V, and stained with 2 µl ethidium bromide (10 mg/ml) to visualize them. Fragment sizes were estimated by comparison with a standard 100 bp DNA marker (Promega).

The maternal species of hybrids was identified by a species-specific RFLP within the mitochondrial D-loop sequence. To develop the method, D-loop sequences of the two megrim species were obtained from GenBank (accession numbers FJ590680-FJ590700 and FJ590730-FJ590750) and aligned with the ClustalW application [45] included in BioEdit. Invariant (monomorphic) regions were visually identified within each megrim species with the BioEdit Sequence Alignment Editor software [46] after sequences alignment. Restriction enzyme targets within the invariant regions of each species were detected with the NEBcutter ver. 2.0 software. The enzyme Dra I recognizes the sequence 5’-TTTAAA-3’ and makes a blunt cut 5’-TTT/AAA-3’ [47]. Such sequence is present in *L. whiffiagonis* and absent in *L. boscii* D-loop sequences.

D-loop amplification was carried out employing the primers D-loopDF (5’-GTCGCCACCATTAACTTATGC-3’) and D-loopDR (5’-CCCAAACTCCCAAAGCTAAG-3’) described by [48]. The amplification mixture, of a total volume of 20 µl, contained 4 µl of 5x Promega Green Buffer, 1.2 µl of 25 mM $MgCl_2$, 2 µl of a 2.5 mM dNTPs mixture, 1 µl of each primer at 20 µM, 0.12 µl of GoTaq polymerase at 5U/µl (Promega), 2 µl of sample DNA and 8.68 µl of bidistilled water. Eight µl of PCR amplification were loaded in a 2% agarose gel stained with 2 µl of 10 mg ml-1 ethidium bromide to verify that only one band was amplified. Then 10 µl of the PCR product were mixed with 2.5 µl of 10x Buffer M (Roche), 0.1 µl of Dra I enzyme (Roche) at 10 ud/µl and 12.4 µl of bidistilled water making a total volume of 25 µl, and incubated at 37ºC for one hour. After incubation, the products were loaded in a 2.5% agarose gel, run at 80 v for 40 min and stained with 2 µl of 10 mg ml-1 ethidium bromide. Fragment sizes were estimated with a standard 100 bp DNA marker (Promega).

4.3 Statistical analysis

The proportions of interspecific hybrids before and after the Prestige accident were compared employing a Chi-square contingency test ($\chi2$) by hand. The null hypothesis (H_0) was that the proportions are similar at a confidence level of 95%.

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Interspecific introgression and changes in population structure in a flatfish species complex after the *Prestige* accident

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Abstract

Oil spills cause aggressive impacts on marine ecosystems affecting immense areas and the species inhabiting therein. If wastes are not cleaned up properly, the remnants will affect local populations for a long time. This work focuses on the long-term impacts of the *Prestige* spillage, occurred off Galician coast (Spain) in November 2002. Model species were two sympatric flatfish, the megrims *Lepidorhombus whiffiagonis* and *Lepidorhombus boscii*. Samples obtained before and nine years after the *Prestige* accident from affected and unaffected areas were genotyped for six hypervariable nuclear markers and for the mitochondrial D-loop sequence. The results revealed a high proportion of post-F1 interspecific hybrids in the area affected, and also increased population differentiation within species likely due to such localized introgression of foreign genes. These changes suggest the appearance of a hybrid zone following the accident and emphasize the need of paying special attention to potential evolutionary impacts of oil spills.

Keywords: Hybrid zone; Introgression; *Lepidorhombus boscii*; *Lepidorhombus whiffiagonis*; Oil spill; *Prestige* accident.

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1. INTRODUCTION

Impacts of human activities on marine ecosystems are varied and of different magnitude: overfishing (Pauly *et al.*, 1998), climate change (Kelly *et al.*, 2010), destruction of coastal areas (reviewed in Jackson *et al.*, 2001) and others.

Amongst the most aggressive impacts are those caused by oil spills, which can be considered large-scale catastrophes *(e.g.,* Piatt *et al.*, 1990; Garcia-Negro et al., 2009). Fuel releases are followed by population declines due to mortality of affected individuals (*e.g.*, Sánchez *et al.*, 2006; Zuberogoitia *et al.*, 2006), changes in populations' gene pool due to mutations (*e.g.*, appearance of new alleles or alteration of its frequencies; Cronin and Bickman, 1998), and altered relationships between species, like for example increased interspecific hybridization (Crego-Prieto *et al.*, 2012a). Some of those changes are theoretically reversible. For example, population reductions may be compensated with migrations from non-affected areas after habitat restoration (Bond and Lake, 2003). However, changes in the genetic pool (*i.e.*, the genetic variants of a population) either due to genetic diversity losses or because of introduction of foreign allospecific genes within a species by hybridization (interspecific introgression) could be irreversible and produce footprints at the evolutionary level.

One of the worst oil spill catastrophes in European waters was caused by the *Prestige* tanker in November 2002 (Albaigés *et al.*, 2006). Before sinking, this tanker released more than 60,000 M-100 type fuel tonnes off the Galician coasts (Northwest Spain), polluting more than 2600 Km of Spanish and French coasts and an area about 30,000 Km2 (Sánchez *et al.*, 2006). Plankton invertebrates (Pérez-del-Olmo *et al.*, 2009), sessile molluscs (Laffon *et al.*, 2006), birds (Zuberogoitia *et al.*, 2006), fishes (Sánchez *et al.*, 2006), mammals, and human health (Zock *et al.*, 2011) were affected by the heavy contamination that followed the accident. The *Prestige* oil was lowly volatile, insoluble in water and viscous (www.cedre.fr). The majority of fuel was recovered by manual methods, but an important part formed aggregates and sunk. It contained very toxic and carcinogenic compounds (Polycyclic Aromatic Hydrocarbons or PAH; Laffon *et al.*, 2006). The remaining fuel in the ship (about 17,000 t) received no treatment. Since it was less dense than the water at that profundity (3600 m depth) (www.cedre.fr), it was slowly released and has been affecting the area and the species nearby since the spillage.

Increased hybridization between flatfish congeners following the *Prestige* oil spill has been recently described (Crego-Prieto *et al.*, 2012a). Hybrid mating was explained either by reduction of spawning areas and/or altered mating behaviour in the zone directly affected by the accident. Spatial barriers in the marine realm are not impassable (Waples, 1998), therefore these hybrids could potentially move into other areas of the species distribution range, affecting as well the relationships between populations within each species.

The impact of the *Prestige* accident could be even more important if the affected species are subjected to additional stress, for example if they are fishery targets (www.fao.org, www.ices.dk). Fishing removes adults from wild populations and thus reduces their reproductive potential (Trippel *et al.*, 1997). This is the case of megrims. *Lepidorhombus boscii* (four-spotted megrim; Risso, 1810) and *Lepidorhombus whiffiagonis* (common megrim; Walbaum, 1792) are very important at commercial level for European fisheries (*e.g.* Pinnegar *et al.*, 2002). Fished together as a single target (ICES WGHMM, 2008), the two megrims are caught by drift or bottom trawl. A number of European countries fish for megrims and, according to FAO (Food and Agriculture Organization of the United Nations, www.fao.org), Spain and the UK contribute the most to total landings (~70% of total) followed by France and Ireland.

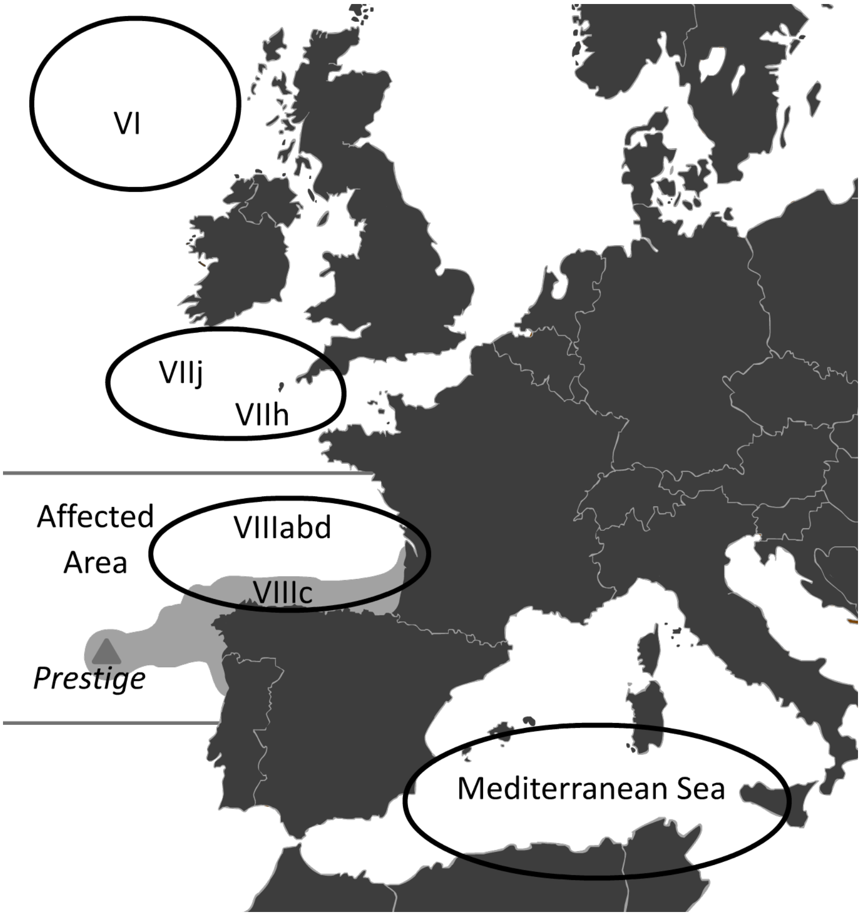


Figure 1
Map of west Europe showing the location of the *Prestige* accident (triangle) and the region affected by the oil spill in the first months (light grey), that was expanded in the following months covering a bigger area (estimated final affected area is delimited by grey lines). Sampling areas are marked with circles and include the names of the corresponding ICES areas.

Regarding population differentiation, the ICES (International Council for the Exploration of the Seas, www.ices.dk) considers three different stocks for *L. whiffiagonis* and *L. boscii* in the following areas: Mediterranean Sea; Southern Bay of Biscay and Iberian basin; Celtic Sea-West of Scotland-Northern Bay of Biscay. One more stock is considered for *L. whiffiagonis* in northern waters: North Sea and Icelandic waters. The Mediterranean stocks are clearly differentiated from the Atlantic ones and seem to be genetically separated from them (Garcia-Vazquez *et al.*, 2006). The northern and southern Atlantic stocks of *L. boscii* seem to exhibit abrupt genetic differences, whereas *L. whiffiagonis* populations would rather follow a model of isolation by distance (genetic and geographic distances being correlated) and significant difference would occur only between distant locations (Danancher and Garcia-Vazquez, 2009). The area affected by the *Prestige* oil spill is located within the distribution of the Southern Bay of Biscay and Iberian basin stock (Fig. 1).

In Crego-Prieto *et al.* (2012a) only one species-specific marker (5S rDNA) was employed to determine interspecific hybridization, and therefore it was not possible to know whether hybrids were of first (F1) of further generations (post-F1, including backcrosses). It was neither known if hybrids remained in the area affected by the *Prestige* or moved to other regions, maybe changing the normal pattern of differentiation among populations for one or the two species (described in Danancher and Garcia-Vazquez, 2009).

This study aims at assessing the genetic impact of the *Prestige* oil spill across the species' distribution of the two *Lepidorhombus* species. Employing population genetic methodology and mitochondrial and nuclear hypervariable loci as genetic markers, we have evaluated the evolutionary impact of the *Prestige* oil spill in European megrims on pre- and post-*Prestige* samples (these obtained nine years after the catastrophe, approximately 4–5 generations for these species). Departure hypotheses were: (a) Post-F1 hybrids will be found in affected populations if hybrids are fertile; (b) genetic diversity will increase in affected populations due to hybridization; (c) population differentiation will increase within each species due to genetic change in the affected areas.

2. MATERIALS AND METHODS

2.1 Samples analyzed

2.1.1 Pre-*Prestige* samples

Megrim samples (gill or muscle tissue stored in 100% ethanol) from northern (ICES VIIj, VIIh and VI; 48 *L. whiffiagonis* and 45 *L. boscii*) and southern North Atlantic areas (ICES VIIIc and VIIIa,b,d; 34 *L. whiffiagonis* and 31 *L. boscii*) were collected from fishing vessels in 2001 (Table 1). The southern areas were later contaminated by the *Prestige* oil spill. Samples were divided into these two groups due to such later primary exposure to the spillage. Some samples have previously been analyzed for microsatellites (Danancher and Garcia-Vazquez, 2009) and mitochondrial DNA (Campo and Garcia-Vazquez, 2010).

Table 1
Microsatellite loci variation in the considered populations of *L. whiffiagonis* and *L. boscii* in a) pre- and b) post-*Prestige* samples. Sample size (n); number of alleles (NA); allelic richness (A.R.); expected and observed heterozygosity (He and Ho); p-value for Hardy-Weinberg equilibrium (HWE).

a		North Stock	South Stock	Mediterranean	Total/Mean
L. whiffiagonis	n	48	34	-	82
	NA	6.83 (4.07)	5.50 (4.85)	-	6.17 (4.46)
	A.R.	5.45 (3.45)	4.82 (3.96)	-	5.14 (3.70)
	Ho (SD)	0.527 (0.281)	0.530 (0,352)	-	0.528 (0.050)
	He (SD)	0.476 (0.235)	0.453 (0.247)	-	0.465 (0.008)
	HWE	0.559	0.388	-	0.474
L. boscii	n	45	31	-	76
	NA	8.83 (10.55)	7.83 (8.95)	-	8.83 (9.75)
	A.R.	6.76 (7.45)	6.91 (7.38)	-	6.83 (7.42)
	Ho (SD)	0.421 (0.312)	0.580 (0.226)	-	0.501 (0.060)
	He (SD)	0.507 (0.251)	0.591 (0.278)	-	0.549 (0.019)
	HWE	0.176	0.257	-	0.216

b		North Stock	South Stock	Mediterranean	Total/Mean
L. whiffiagonis	n	40	13	45	98
	NA	5.83 (5.19)	5.83 (3.66)	6.33 (2.94)	6.00 (3.93)
	A.R.	3.83 (2.79)	5.62 (3.39)	4.05 (1.83)	4.50 (2.67)
	Ho (SD)	0.545 (0.125)	0.705 (0.331)	0.539 (0.242)	0.595 (0.233)
	He (SD)	0.568 (0.183)	0.714 (0.221)	0.524 (0.229)	0.602 (0.211)
	HWE	0.176	0.387	0.330	0.298
L. boscii	n	18	64	46	128
	NA	6.33 (6.44)	8.67 (6.53)	6.00 (7.87)	7.00 (6.95)
	A.R.	5.13 (4.59)	4.97 (3.07)	3.80 (3.89)	4.63 (3.85)
	Ho (SD)	0.656 (0.243)	0.547 (0.261)	0.464 (0.258)	0.555 (0.254)
	He (SD)	0.642 (0.247)	0.578 (0.229)	0.462 (0.243)	0.561 (0.240)
	HWE	0.448	0.200	0.297	0.315

2.1.1 Post-*Prestige* samples

In 2011, nine years after the oil spill, samples of the two megrim species were collected from fishing vessels and research cruises operating in the northern area unaffected by the oil spill (ICES VIIh, VIIj and VI; 40 *L. whiffiagonis* and 18 *L. boscii*), and from the affected southern area (ICES VIIIc and VIIIabd areas; 13 *L. whiffiagonis* and 64 *L. boscii*) (Fig. 1, Table 1). Mediterranean samples were directly purchased at landings from Spanish and Italian coasts in the same year. Samples from the most affected area (VIIIc) have been already genotyped for the 5S rDNA (Crego-Prieto et al., 2012a).

All samples are stored in the laboratory of the research team at the University of Oviedo (Spain), available under request.

2.2 DNA analyses

DNA was extracted from 1 mm^3 piece of muscle sample following a Chelex-based protocol (Estoup *et al.*, 1996). Genotypes for the microsatellite loci lepi-P8, lepi-P21, lepi-P29, lepi-P34, lepi-P38 and lepi-P40 were obtained following the PCR-based protocol described by Danancher and Garcia-Vazquez (2009). Genotypes for the 5S rDNA locus were obtained following the PCR-based protocol described in Crego-Prieto *et al.* (2012a).

The maternally inherited mitochondrial D-loop sequence, which is different in the two species, was obtained in order to confirm the maternal species of hybrids. Sequences were obtained by PCR amplification and sequencing following Campo and Garcia-Vazquez (2010). Some sequences had been obtained previously by these authors (GenBank Accession Nos. FJ590665-750) and others were obtained in this work. They were uploaded to the GenBank and are available under the Accession Nos. KC550009-031, KC684890-905 and KC700602-609.

2.3 Data analysis

2.3.1 Genetic assigment to a species or hybrid status

We employed the NewHybrids software ver. 1.0 (Anderson, 2002; Anderson and Thompson, 2002) for determining the species status of the samples based on microsatellite genotypes. The software computes the posterior probability for an individual to belong to a pure species, F1 (first-generation hybrid), backcross (offspring of a cross F1 × parent species) and F2 (second hybrid generation) categories. Four different runs of 300,000 “sweeps” were performed after a burn-in of 100,000 “sweeps” (a “sweep” is a standard iteration of the MCMC algorithm). This software also gives the direction of the hybrid cross or backcross, that was confirmed based on maternally-inherited mitochondrial D-loop sequences.

2.3.2 Estimates of genetic variation and population differentiation

The following parameters of genetic diversity were measured for each population and species: mean number of alleles (genetic variants) per locus (Na), allelic richness (AR), mean observed and expected heterozygosity

(Ho and He), and departures from Hardy–Weinberg population genetic equilibrium. These parameters were calculated with the software FSTAT (Goudet, 1995). The same program was employed to compare groups of samples (*e.g.* pre- versus post-*Prestige*) for genetic diversity. Two-sided tests were made with 10,000 permutations. Genetic differentiation between populations was assessed from pairwise FST values, calculated with the program ARLEQUIN ver. 3.0 (Excoffier *et al.*, 2005) through 10,000 permutations.

2.3.3 Determination of population structuring

The number of genetic units (populations) of each species present in the dataset and their admixture degree were estimated from the microsatellite dataset by genotype-based inferences using a Bayesian approach with the STRUCTURE software ver. 2.3.3 (Pritchard *et al.*, 2000). The optimal value of K (number of different genetic units) was estimated for pre- and post-*Prestige* samples of each species separately, running K from 1 to 5 (the samples analyzed correspond to three different stocks but subdivisions could theoretically occur) ten independent times. Admixture model (each individual may have mixed ancestry and draw some fraction of its genome from each of the K clusters) and correlated allele frequencies were chosen as settings, with a burn-in period of 30,000 steps followed by 300,000 Monte Carlo Markov Chain (MCMC) steps to ensure convergence. The methods of ‘Ln P(D)’ (Pritchard *et al.*, 2000) and ‘ΔK’ (Evanno *et al.*, 2005), were used to determine the best K that fitted our data. Often true K is identified with the maximal value of ‘Ln P(D)’ (Evanno *et al.*, 2005), but a clear and easier interpretation is obtained with ‘ΔK’.

2.3.4 Statistical analyses

Comparison between samples for the proportion of pure species and hybrids was done employing contingency Chi-square tests, calculated manually.

3. RESULTS

3.1 Interspecific introgression

The results obtained with the NewHybrids software (Fig. 2a) indicate that before the Prestige accident the two species were clearly separated in all their Atlantic distribution with only a few post-F1 hybrids in the northern stock. Some originated from *L. boscii* mother and others from *L. whiffiagonis* females as inferred from D-loop sequences; therefore hybrid crosses occurred in the two directions. After the *Prestige* oil spill the species composition changed slightly in the northern group because hybrids were not found with NewHybrids (Fig. 2b). One individual with *L. whiffiagonis* D-loop was assigned to the species *L. boscii* based on microsatellite loci, revealing that it was of hybrid origin. In the area affected by the spillage, however, the situation changed drastically with a high proportion of post-F1 hybrids: 39% and 10% issued from *L. boscii* and *L. whiffiagonis* mothers, respectively. Differences between affected and unaffected areas for hybrid proportion were indeed highly significant (Contingency Chi-Square test for two classes, hybrids vs. non-

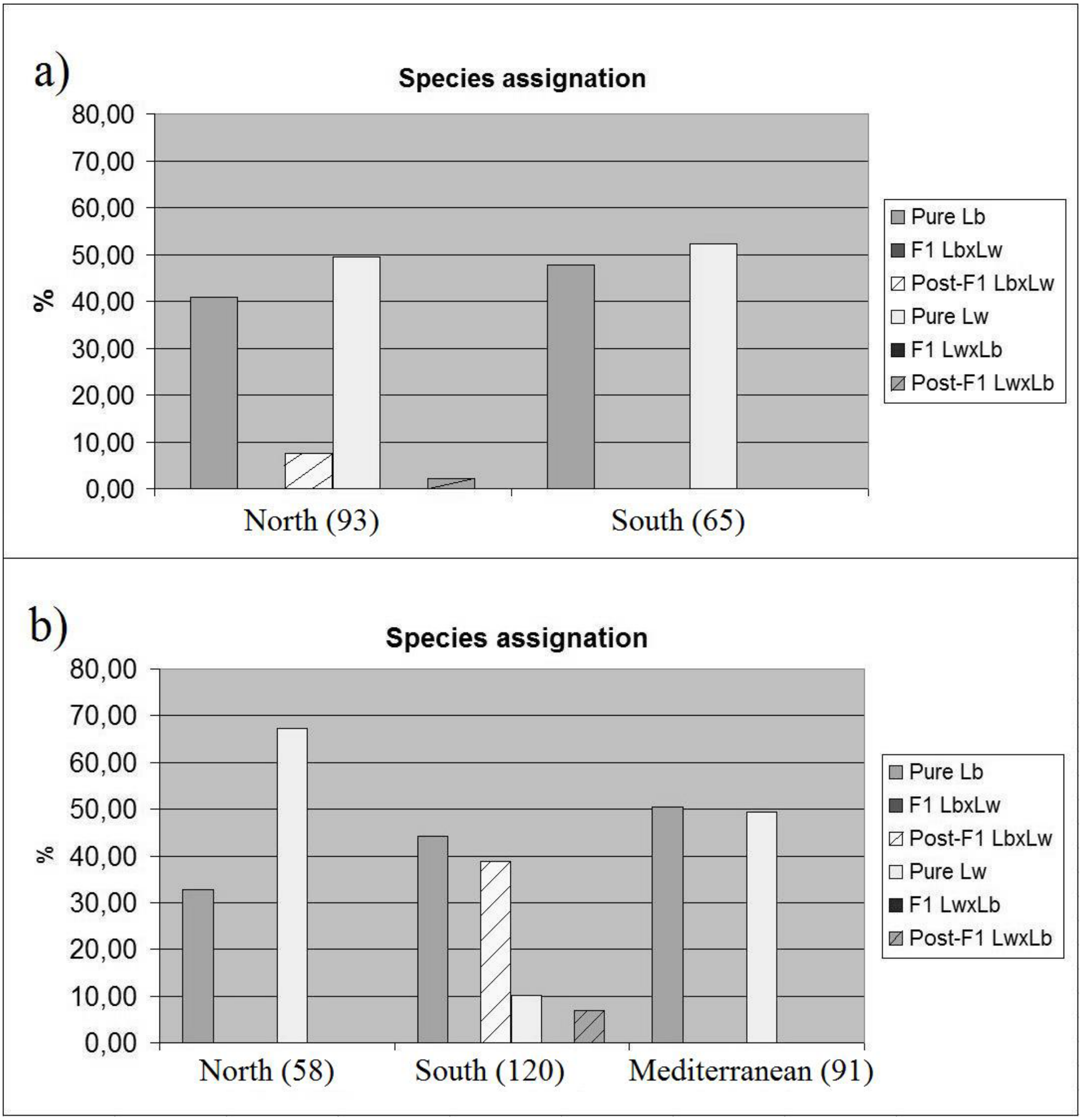


Fig. 2
Species assignation of megrims in the studied European marine regions in (a) pre- and (b) post-*Prestige* samples. Results are presented as the percent of individuals of each type: pure species, F1 and post-F1 hybrids of each cross direction (mother species first, identified from mitochondrial DNA) for the three stocks: north, south and Mediterranean.

hybrids, = 55.414, 1 degree of freedom, $P < 0.001$). Hybrids were not detected from Mediterranean samples.

3.2 Changes in population structuring within species

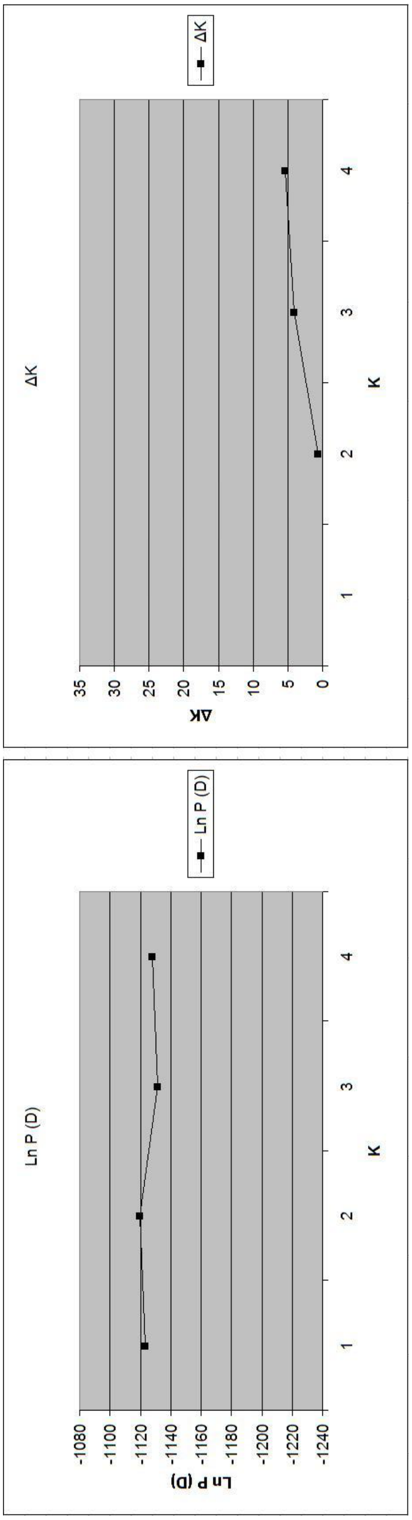
Before the *Prestige* accident, values of genetic diversity such as the number of alleles per locus and allelic richness (Na and AR respectively) were higher in general for *L. boscii* than for *L. whiffiagonis* populations (Table 1). Megrim populations did not exhibit deviations from Hardy–Weinberg equilibrium in any case (HWE; $P > 0.05$). After the *Prestige* accident the global microsatellite variation did not change for these species. Diversity parameter values (Na, AR, Ho and He) were not significantly different for pre- and post-Prestige samples for any species (two-sided *P*-values > 0.05).

On the other hand, the STRUCTURE results obtained before the oil spill for *L. whiffiagonis* were consistent with one only genetic cluster (Fig. 3a and c), whereas *L. boscii* northern and southern populations were separated in two different genetic units (Fig. 4a and c). After the accident, *L. boscii* conserved a similar structuring in two genetic clusters (Fig. 4b and d). Highly significant F_{ST} values were found between the two *L. boscii* populations before ($F_{ST} = 0.574$, $P < 0.0001$) and after the accident ($F_{ST} = 0.284$, $P < 0.0001$), as expected from STRUCTURE results. However, the situation changed for *L. whiffiagonis* (Fig. 3b and d), for which two well differentiated genetic units were obtained after the Prestige: the affected and the unaffected areas were genetically differentiated, although some individuals exhibited mixed membership. This was confirmed by highly significant F_{ST} value of 0.306 ($P < 0.0001$) between these areas.

4. DISCUSSION

The results of this study reveal changes of potential evolutionary significance in megrims after the *Prestige* oil spill. The first and most evident one was high interspecific introgression in the affected populations of the two species (Fig. 2). Hybrids previously detected with a single species-specific locus in this zone (Crego-Prieto *et al.*, 2012a) have been revealed here to be post-F1, *i.e.* backcrosses between F1 hybrids and the two pure species. The high proportion of hybrids (>45%) exceeds introgressive hybridization values reported so far in fish (*e.g.*, Hartley, 1996; Castillo *et al.*, 2008; Machado-Schiaffino *et al.*, 2010). The fact that all hybrids are of post-F1 status strongly suggests that interspecific crosses occurred at the time of the accident but were not recurrent. *L. boscii* matures after 1.5 years (Perry *et al.*, 2005). For *L. whiffiagonis*, which exhibits similar life-history traits, a similar age of first maturation can be estimated. Therefore, approximately four or five generations have passed for these species since the accident. Occurrence of post-F1 individuals also demonstrates that hybrids are fertile. Although hybrids between sympatric marine fish are not unusual (revision in Mallet, 2005), their frequency is normally lower than 10%, like found in the northern area in our study. The much higher proportion of hybrids found after the spillage in the affected area suggests that interspecific barriers have been altered

(a)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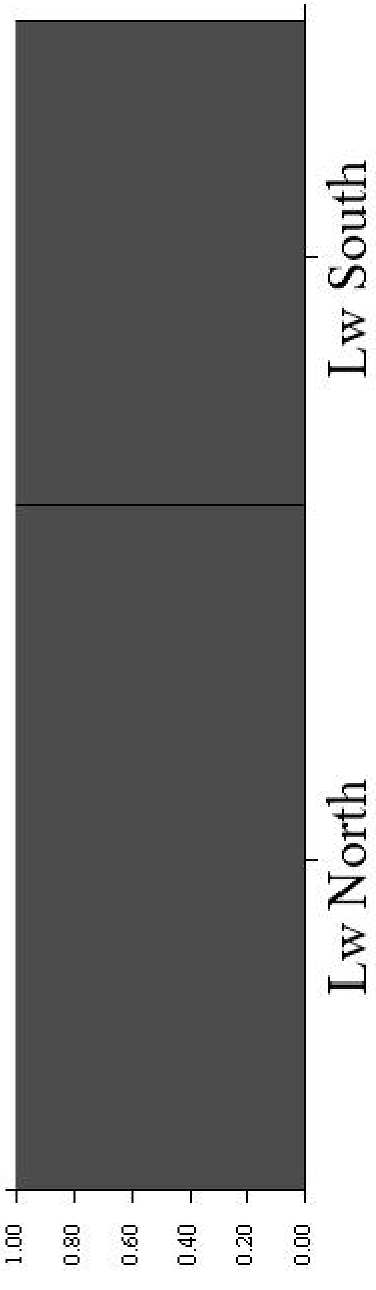
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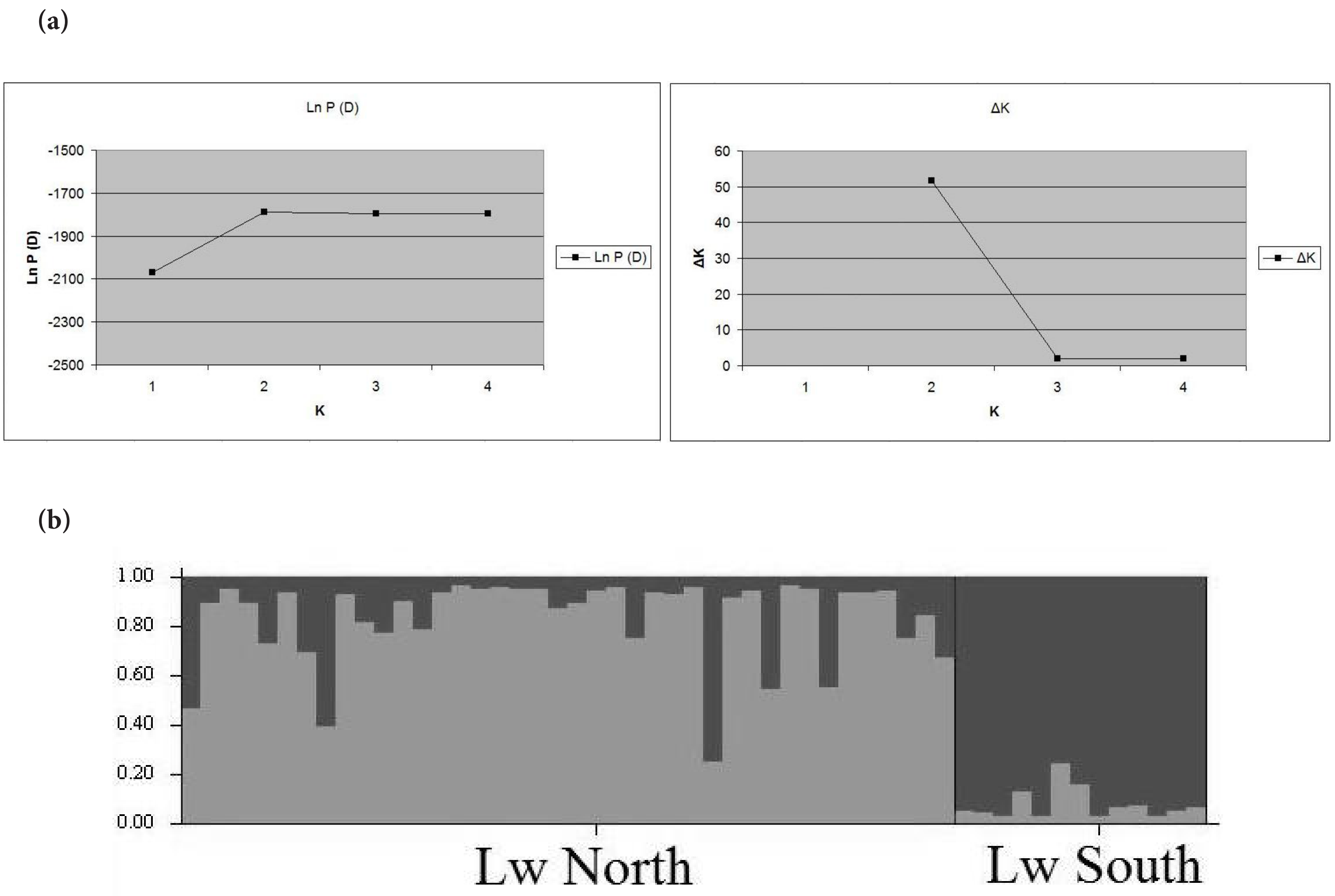
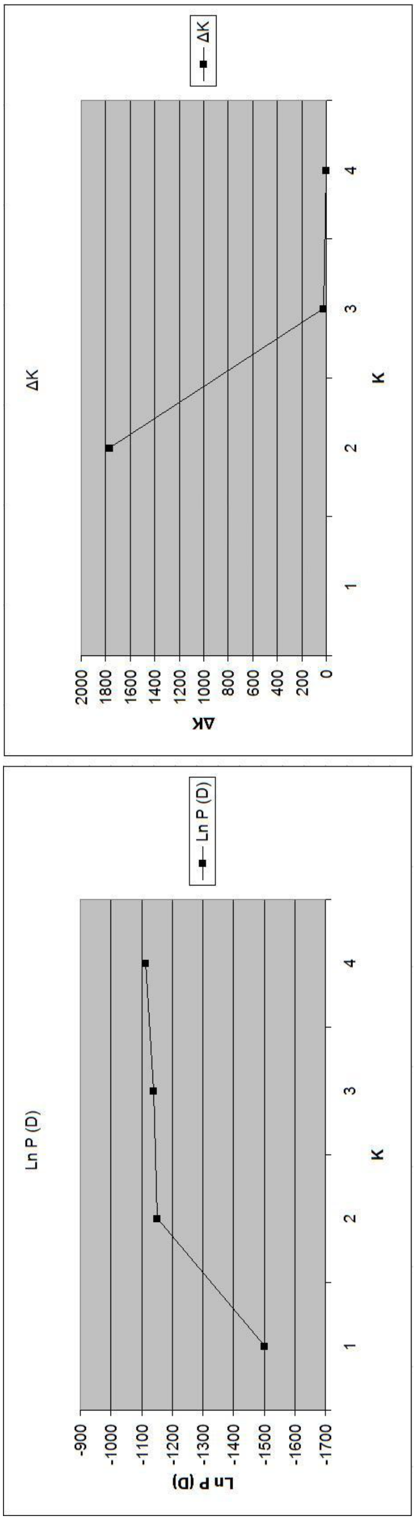


Fig.3
Estimation of population clustering level from *Lepidorhombus whiffiagon*is microsatellite genotypes following Pritchard *et al.* (2000) (left) and Evanno *et al.* (2005) (right) criteria, and individual membership in pre-*Prestige* (a and c respectively) and post-*Prestige* (b and d) samples.

(a)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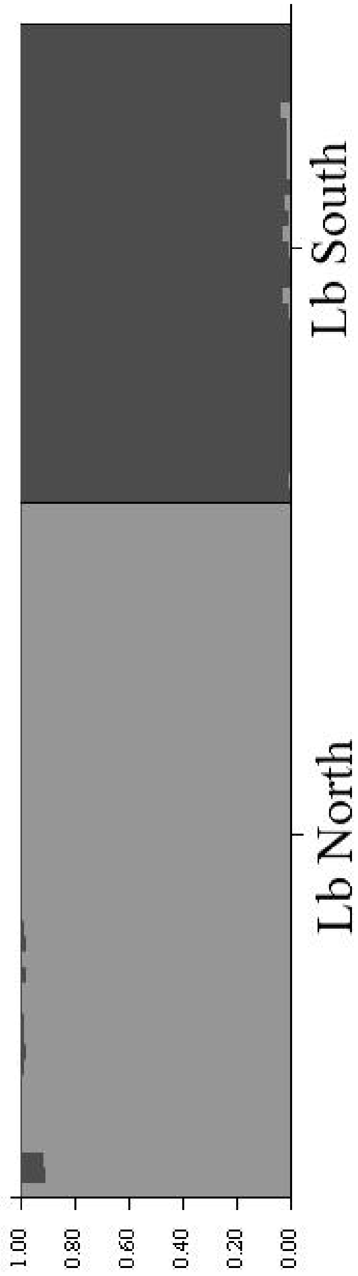
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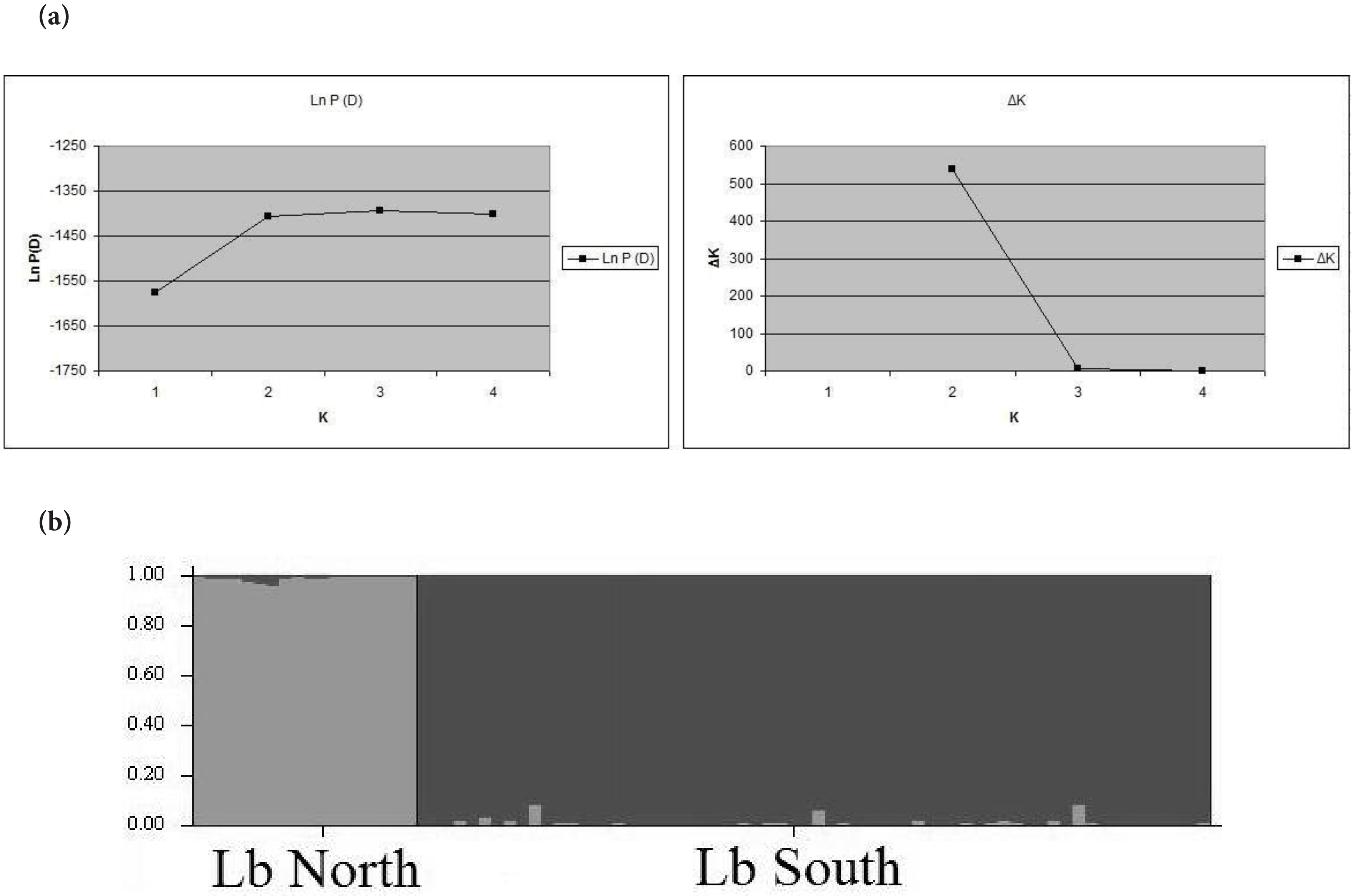

Fig.4
Estimation of population clustering level from *Lepidorhombus boscii* microsatellite genotypes following Pritchard *et al.* (2000) (left) and Evanno *et al.* (2005) (right) criteria, and individual membership in pre-*Prestige* (a and c respectively) and post-*Prestige* (b and d) samples.

there. Scribner *et al.* (2001) identified the habitat loss (or alteration of spawning areas) as one of the main factors that promote hybridization among species. The current scenario could be interpreted as a hybrid zone spatially and temporally compatible with the *Prestige* accident, which could be considered the cause of this evolutionary significant change.

Hybrid zones are tension zones very vulnerable to changes in environmental, biological or stochastic factors (Barton and Hewitt, 1985). Therefore, long-term evolution of this hybrid zone is difficult to predict. It may persist for a long time, or be progressively diluted due to migration and/or perhaps reduced fitness of post-F1 hybrids, as it happens in other fish (*e.g.*, Garcia-Vazquez *et al.*, 2004). Other studies have demonstrated the long-term effects of oil spills. Sanders *et al.* (1980) determined physiological and behavioral abnormalities after 5 years of exposure to *Florida*'s oil (sunk in Massachusetts, 1969) in different species. Piatt and Anderson (1996) showed population declines, reduction of breeding success and alteration of breeding time in common murres after the *Exxon Valdez* oil spill (sunk in Alaska, 1989); and 5 years later there was still a shift in the marine ecosystem, changing both the marine community composition and species ecology. Likewise, long-term changes could also occur in the case for the *Prestige* oil spill, and the hybrid zone could be one of them.

Regarding population structuring within species, the most noticeable change was increased genetic differentiation between the affected and the unaffected areas for *L. whiffiagonis* (Fig. 3b), as expected. Change in population structuring was not detected, however, for *L. boscii*. This species exhibits genetic differences between populations in the Atlantic Ocean (Danancher and Garcia-Vazquez, 2009), and the affected and unaffected areas were already significantly different before the *Prestige* oil spill. Differentiation between them is not expected to be erased after the change occurred in the affected area.

Contrary to our expectation, significant changes of within-population variation did not occur following interspecific introgression. It is known that megrim exhibited high mortality due to the oil spill (Martínez-Gómez *et al.*, 2006). Thus, it is possible that the loss of within-population genetic diversity caused by such population reduction was compensated with the new diversity introduced via interspecific hybridization.

The results found in this study also emphasize the convenience of using multiple markers for hybridization studies. Those based only on one genetic marker, since although valuable as a first call of attention, they have limitations due to the impossibility of identifying post-F1 hybrids (Crego-Prieto *et al.*, 2012a).

As a final remark, potential evolutionary impacts derived from increased introgression and changes in population structuring should be taken into account when facing catastrophes in the marine environment (whether caused by human activities or not). Applying protective or restorative management of the affected species would be a priority for re-establishing the equilibrium in complexes of sympatric species.

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Zuberogoitia, I., Martinez, J.A., Iraeta, A., Azkona, A., Zabala, J., Jimenez, B., Merino, R., Gomez, G., 2006. Short-term effects of the prestige oil spill on the peregrine falcon (Falco peregrinus). Marine Pollution Bulletin 52, 1176-1181.hybrids, = 55.414, 1 degree of freedom, P < 0.001). Hybrids were not detected from Mediterranean samples.

Estuaries and Coasts

Cytological and population genetic changes in Northwestern Iberian mussels after the *Prestige* oil spill

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Abstract

The ecological catastrophe produced by the *Prestige* oil spill (November 2002) caused severe damage in both North Spanish and French coastal communities. Wild mussel populations of *Mytilus galloprovincialis* in a zone with marginal introgression of *M. edulis* were affected at all levels, from high DNA damage to increased polycyclic aromatic hydrocarbons content in tissues. In this article we describe cytological and genetic changes of wild mussel populations from the northwestern Iberian coast following the catastrophe. The micronucleus test was employed as an indicator of cytological damage, and the Barcoding mitochondrial cytochrome oxidase I (COI) and the nuclear Glu-5' genes were analyzed for determining the species and assessing population genetic diversity. Rapid recovery from cytological damage was found. However, reduced variation at mitochondrial sequences in the most exposed areas and reduction of *M. edulis* traces in the regional genetic pool suggest long-term impact that may result in evolutionary changes. The results highlight the need of adopting more strict measures in order to prevent this type of accidents and avoid long-term effects on wild populations.

Keywords: Micronucleus test; *Mytilus edulis; Mytilus galloprovincialis*; *Prestige* oil spill; population genetic diversity; recovery; interspecific introgression.

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1. INTRODUCTION

The year 2012 was the tenth anniversary of the *Prestige* oil spill catastrophe, one of the worst ecological accidents in the history of Europe (Albaiges *et al.* 2006). The *Prestige* tanker sank off the Galician coast (Northwest of Spain; 42°10’8”N 12°3’13”W) in November 2002, causing an ecological disaster of fuel oil contamination in the Atlantic European coasts and an enormous damage to local Spanish economy (Garcia-Perez 2003). Approximately 20,000 tonnes of fuel were dumped into the ocean at the time of the accident and 40,000 more in the six dramatic following days (Albaiges *et al.* 2006; Sanchez *et al.* 2006). After that, the tanker sank with a large quantity of oil still on board (approx. 16,000 tonnes) to the depth of 3,600 meters, 250 Km off the Spanish coast. In contact with the water, emulsification of the fuel oil occurs and its volume can be incremented up to three times (www.cetmar.org/?lang=en). The oil spill affected the neighboring regions (Fig. 1), expanding along the Cantabrian coast and the Bay of Biscay in a few months (European Topic Centre on

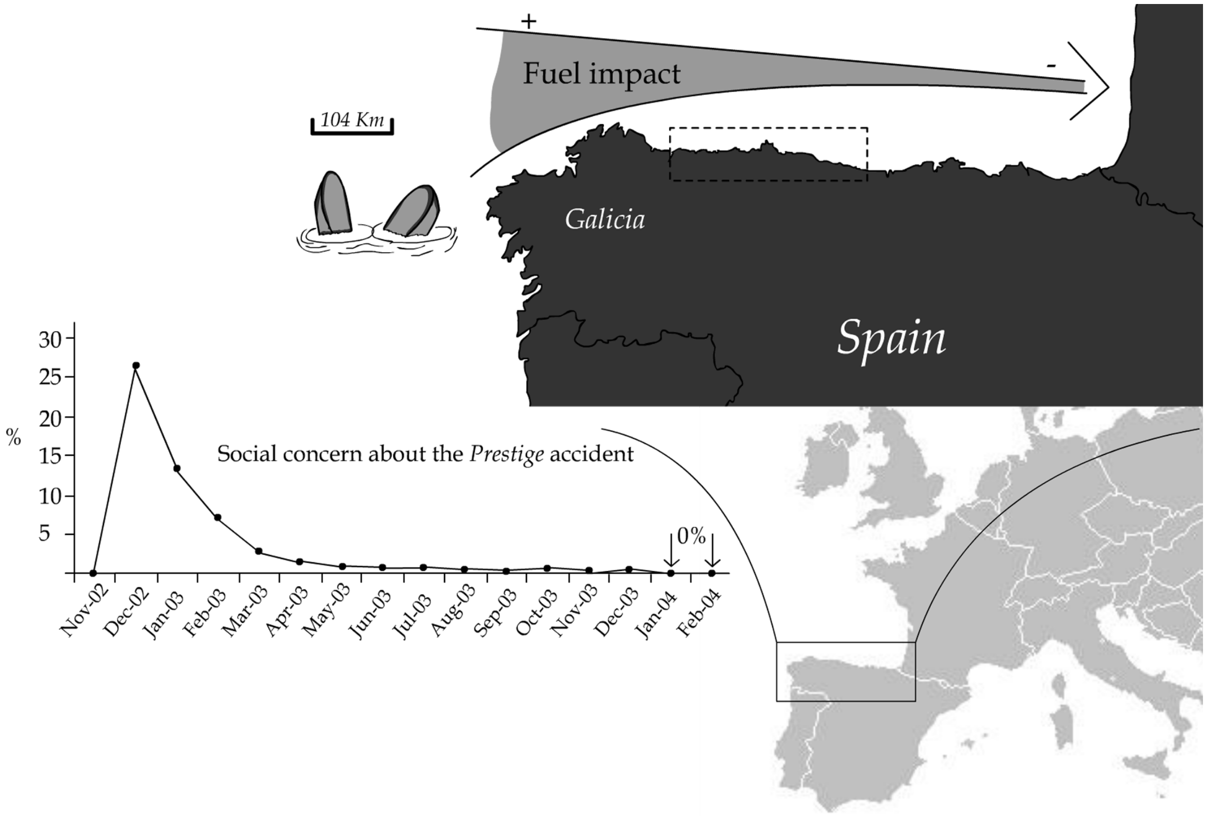

Fig. 1
Map showing the location of the *Prestige* accident, the coastal region sampled (dotted rectangle) and the evolution of the public concern about *Prestige*’s impacts in Spain following the catastrophe. The arrow reflects the relative impact of the fuel in the areas affected (higher in the West).

Spatial Information and Analysis (EIONET, http://sia.eionet.europa.eu/en_Prestige); Pérez-del-Olmo *et al.* 2007). More than 2,500 km of Spanish and French shoreline were affected (Martínez-Gómez *et al.* 2006) by the fuel, also called the Black Tide. The accident generated a public outcry that mobilized approximately 300,000 volunteers (the White Tide) from the surrounding countries (mainly from Spain). They collaborated cleaning the fuel oil all along the coast, not without risking their health (Suarez *et al.* 2005; Carrasco *et al.* 2006; Aguilera *et al.* 2010; Zock *et al.* 2012). About 141,000 tonnes of oily waste were collected in Spain and 18,300 tonnes in France.

Many marine species were affected by the *Prestige* catastrophe, from invertebrates (Perez-del-Olmo *et al.* 2009) to mollusks (Piñeira *et al.* 2008; Soriano *et al.* 2006) and fishes (Martinez-Gomez *et al.* 2006; Crego-Prieto *et al.* 2012). Crude oil from the *Prestige* spill was detected on cultured mussels in the Galician coast (Peteiro *et al.* 2007) and caused the obligated closure of most shellfish farms (Suris-Regueiro *et al.* 2007). The contamination quickly arrived to the neighboring regions within the Cantabrian Sea (Fig. 1), where mussels are not farmed but traditionally harvested for local consumption. The Cape Peñas divides the Cantabrian coast in two sections (West and East) of different environmental and biogeographic conditions (Dickson and Hughes 1981; Rios *et al.* 1987; Muñoz-Colmenero *et al.* 2012), constituting therefore a transitional area of special interest for conservation. While recovery of affected species has been widely studied in Galicia and the Basque

Table 1
Trends (persistence / recovery of pollution effects) detected in species affected by the *Prestige* oil spill up to ten years after the arrival of fuel oil.

Trend	Organism	Taxonomic level	Bioindicator	Zone	Reference
Recovery	Mussels	Mollusks	Tissue-level biomarkers	Galicia, Basque Country	Cajaraville *et al.* (2006)
Recovery	Megrim Shrimp	Fish Crustaceans	Stock abundance	Basque Country	Sanchez *et al.* (2006)
Recovery	Mussels	Mollusks	PAH content	Galicia	Soriano *et al.* (2006)
Persistence	Polychaetes	Worms	PAH content	Galicia	Morales-Caselles *et al.* (2008)
Persistence	Fish parasites	Protozoan, prokaryotes	Composition of parasite communities	Galicia	Perez-del-Olmo *et al.* (2008)
Persistence	Periwinkle (Snail)	Mollusks	Shell morphology Population genetic variability	Galicia	Piñeira *et al.* (2008)
Persistence	Megrims	Fish	Interspecific hybridization	Northwest Spain	Crego *et al.* (2012)
Partial recovery	Mussels	Mollusks	Five integrative indices	Galicia, Basque Country	Marigómez *et al.* (2013)

Country (Table 1), the intermediate region has not been a main focus of attention by researchers on *Prestige*'s impacts to date.

Pollutants affect organisms at many levels (*e.g.* Di Giulio *et al.* 1993; Myers *et al.* 1998). The micronucleus (MN) test measures cytological damage, namely appearance of micronuclei and other abnormal nuclear shapes. The MN test has been widely applied for assessing pollution impact in water ecosystems employing mussels as target species (*e.g.* Dailanis *et al.* 2003; Izquierdo *et al.* 2003; Baršienė *et al.* 2004; Gorbi *et al.* 2008). Populations may recover rapidly after the impact and return to normal cytological levels once the pollution has been removed (*e.g.* Machado-Schiaffino *et al.* 2009). On the other hand, permanent -or at least long-term- effects of pollutants may occur at population genetic level in diverse ways. If a population is drastically reduced in size due to heavy contamination, it may exhibit diversity losses (*e.g.* Ma *et al.* 2000). In sympatric species, pollution may alter their reproduction and hybrids may increase, as was the case of some flatfish following the *Prestige* spill (Crego-Prieto *et al.* 2012); this would produce a deep evolutionary trace of interspecific introgression between the affected species.

Wild mussel populations from the northwest Iberian coast affected by the *Prestige* are principally from the species *Mytilus galloprovincialis*. The southern limit of M. edulis is located near the River Bidasoa's mouth in the Spanish-French border (*e.g.* Sanjuan *et al.* 1994), and there is a hybrid zone between this species and *M. galloprovincialis* along the Atlantic French coast and the British Islands (*e.g.* Skibinski *et al.* 1983; Coustau *et al.* 1991). Some introgression between the two species occurs in the Iberian coast. Before the *Prestige* accident, wild *Mytilus galloprovincialis* populations in the Atlantic Iberian coasts contained 8-11% genes of *M. edulis* (Daguin *et al.* 2001). The Cantabrian coast sheltered a panmictic population that was part of a large metapopulation comprising Western Atlantic and Alboran Sea areas (Diz and Presa 2008). About the environmental status, abnormal mussel MN records before the *Prestige* showed that in some areas wild mussel populations were affected by urban and industrial pollution, but in clean zones they were cytologically normal (Izquierdo *et al.* 2003).

The effect of the *Prestige* accident on mussels was enormous. Employing an integrative battery of biomarker indices, Marigomez *et al.* (2013) demonstrated a recovery trend after 2004, although healthy ecosystem condition had not been recovered yet in 2006. These authors did not study the possible impact of the pollution on interspecific introgression.

In this work we have monitored cytological damage in wild mussels from the central-occidental Cantabrian coast when the fuel oil arrived there (February-March 2003) and in the following months. Eight years after the catastrophe, mussels were monitored again for MN and also analyzed at DNA level (mitochondrial and nuclear DNA markers). The objective was to assess cytological recovery and detect possible *Prestige* impacts at population genetic level and on interspecific introgression, of potential evolutionary significance.

2. MATERIAL AND METHODS

2.1 Social indicators

Indicators of Spanish public concerns can be found at the web page of the CIS (Spanish National Centre of Sociological Studies; www.cis.es/cis/export/sites/default/Archivos/Indicadores/documentos_html/TresProblemas.html). This organization makes regular phone surveys of the three main concerns of Spanish random citizens older than 18 years, taking a minimum of 2,500 individuals as sample size. The issue "Impact of the *Prestige* accident" was included starting on November 2002.

2.2 Sample collection

For molecular analyses, the following sampling sites (from West to East) were considered: rocky beaches at Ortigueira, Figueras, Porcia, Luarca, San Pedro, Gijon, Colunga, Toró and La Franca. For the analyses, sites were classified into three groups differentiated by the approximate first date of fuel oil arrival: West (Ortigueira, Figueras, Porcia), Centre (Luarca, San Pedro, Gijon), East (Colunga, Toró, La Franca). First arrival of pollutant occurred in February 2003 in the western area; March 2003 in the centre; and in successive and irregular oil flocks in the East during the following months. The affected areas were manually cleaned by staff of the Regional Administration assisted by thousands of volunteers, starting just after the fuel oil arrival along spring 2003.

For a cytological study, thirty six adult *M. galloprovincialis* had been obtained two years before the *Prestige* oil spill (Izquierdo *et al.* 2003) sampled randomly at the Gijon outskirt's rocky beach covering an area of approx. 500 m2. These samples were here considered as a pre-*Prestige* reference. Post-*Prestige* mussel samples were obtained from the West, centre and East areas in February, March and November 2003. Thirty six samples were taken from each area at each sampling time for cytological analysis, regularly distributed among locations. A further sampling was carried out eight years after the accident, in November 2010. In addition to the individuals sampled for cytological analysis, up to 175 mussels (14-20 per location) were taken for DNA analysis.

2.3 Cytological analysis

For the cytological analyses, mussels were kept in marine water during the transport to the laboratory (around 20 min) and immediately sacrificed. We analyzed micronuclei (MN) in the subpopulation of cells prevailing in gill tissue. A portion of gill was removed using tweezers, dragged along a slide obtaining a single layer of well-spread cells, and then allowed it to dry for a few minutes. Two slides per individual were prepared. Staining procedure followed Izquierdo *et al.* (2003). Briefly, slides were sequentially stained with May-Grünwald for 2 min; May-Grünwald:distilled water 1:1 for 3 min; and Giemsa:distilled water 1:6 for 10 min; then rinsed with distilled water, allowed to dry, and mounted with Eukitt. From each individual, 1,000 main gill cells (500 per slide whenever possible) were scored under 1,000x magnification to determine the frequency of cells with MN. Coded and randomised slides were scored using a blind review by a single observer.

2.4 DNA analysis

DNA was extracted following a standard resin-based protocol as in Muñoz-Colmenero *et al.* (2012). The Barcoding COI sequence region was PCR amplified using the primers HCO2198 and LCO1490 designed by Folmer *et al.* (1994) with the conditions described therein. PCR products were visualized and purified from a 2% agarose gel employing a Promega kit (Wizard SV Gel and PCR Clean-Up System). DNA fragments were sequenced using an ABI Prism BigDye Terminator Cycle sequencing protocol on an ABI Prism 3130 Automated Sequencer at the Unit of Genetic Analysis at the University of Oviedo. Sequences were visualized and edited with

Table 2
Genetic variation of mussel populations in the considered region. N, sample size; *M. edulis* genes, introgression of *Mytilus edulis* (in % of individuals with marker alleles at the locus Glu-05); NH, number of haplotypes; Hd, haplotypic diversity (S.D., standard deviation); π, nucleotide diversity; k, mean number of nucleotide differences between pairs of sequences. R2 parameter, from Onsins and Rozas (2002); Tajima's D, from Tajima (1989); Fu's Fs, from Fu (1997) and Fu & Li's D, from Fu and Li (1993), estimated with DnaSP v5 (Librado and Rozas 2009).

	West	**Centre**	**East**
N	54	56	65
M. edulis genes	5.26%	5.08%	2.99%
***M. galloprovincialis* variation**			
NH	12	17	18
Hd (S.D.)	0.847 (0.079)	0.957 (0.024)	0.905 (0.038)
π (S.D.)	0.005 (0.001)	0.011 (0.002)	0.008 (0.002)
k	2.905	6.307	4.837
Demographic indicators			
Private haplotypes	7	10	11
R2	0.091	0.078	0.055
Tajima's D	-1.842*	-1.129	-1.939*
Fu's Fs	-5.402	-5.487	-6.687
Fu & Li's D	-2.418*	-1.396	-2.616*

BioEdit Sequence Alignment Editor software (Hall 1999), aligned with the MUSCLE program included in the MEGA package (Tamura *et al.* 2011) and compared with international databases in the program nBLAST within NCBI (www.ncbi.nlm.nih.gov/) for species assignation (based on >99% sequence similarity).

The nuclear species-specific gene Glu-5', which encodes for a mussel polyphenolic adhesive protein, was amplified with the primers and protocol described by Rawson *et al.* (1996). The alleles obtained for *M. galloprovincialis* and *M. edulis* differ in size being 200/300 and 380 nucleotides, respectively. The amplification products were visualized in 2% agarose gels.

2.5 Statistical analyses

The frequencies of cells with MN were expressed per 1,000 (‰). After testing for homogeneity of variances, Mann-Whitney U test (significant differences between groups established at $p < 0.05$) was employed to compare nuclear abnormalities frequencies between paired samples (seasons, samples taken after the oil spill). A global Kruskal-Wallis test was employed for comparing MN frequencies of the three samples obtained the year after the oil spill. Analyses were run in PC computers using the SPSS 8.0 program (SPSS Inc.).

The genetic diversity of *M. galloprovincialis* in the three considered areas within the region (West, centre and East) was estimated based on COI gene variants calculating the number of haplotypes (Nh), haplotype (Hd) and nucleotide (π) diversity and the average number of nucleotide differences (k) using the program DnaSP v5 (Librado and Rozas 2009) (Table 2). In addition, estimates of population expansion as Tajima's D, Fu's Fs and Fu and Li's D were calculated using the same software. Negative values of these parameters are considered signals of population expansion. Furthermore, the R2 parameter described by Ramos-Onsins and Rozas (2002) for detecting changes in effective population size (Ne) was estimated employing also the program DnaSP v5 (Librado and Rozas 2009). The degree of change in Ne is proportional to R2.

A Mann-Whitney U-test (Sokal and Rohlf 1995) was used to assess statistical significance of differences in genetic diversity measures: Nh, Hd and π. FST estimates of between-population differentiation were computed with the ARLEQUIN software version 3.5 (Excoffier *et al.* 2005), based on differences of haplotype frequencies. Haplotype networks of the three areas were constructed with the program Network 4.610 (Bandelt *et al.* 1999).

The frequency of *M. edulis* genes introgressed into the *M. galloprovincialis* genetic population pool was calculated in %, considered the individuals which exhibited either a COI sequence of M. edulis or Glu-5' *M. edulis* alleles. The statistical significance of the difference between this percentage and previous estimates for Iberian coasts (Daguin *et al.* 2001) was checked employing a z test implemented in the SPSS 8.0 program (SPSS Inc.).

3. RESULTS

3.1 Cytological abnormalities

Figure 2 shows the averages of MN frequencies found in the sampling sites considered within the region. The central sampling area serves as a control because previous data on MN were available (Izquierdo *et al.* 2003). Micronuclei counts on *M. galloprovincialis* significantly increased there after the arrival of the fuel contamination in March 2003 (Kruskal-Wallis test: $H = 10.75$, $P = 0.0046$), up to 10.5 ± 5.21 (Mann-Whitney U of 192 with $P = 0.045$). After finishing the area cleaning (November 2003) the average MN decreased significantly down to 4.05 ± 0.91 (Mann-Whitney $U = 732$, $P = 0.0029$), a value not significantly different from that found in the control samples. Finally, in November 2010 these cytogenetic abnormalities were 5.23 ± 1.8, similar to initial control records. At the West of the sampled region the petrol arrived earlier and local mussels exhibited significantly higher MN counts than in the control area (8.91 ± 4.34; $p < 0.05$) in February 2003. This value decreased after the cleaning and this trend continued in the following months reaching normal values of 4.92 ($p < 0.05$) in November 2003. Finally, at the East of the region the pollution arrived intermittently and in lower quantities. Significant changes in MN counts were not observed

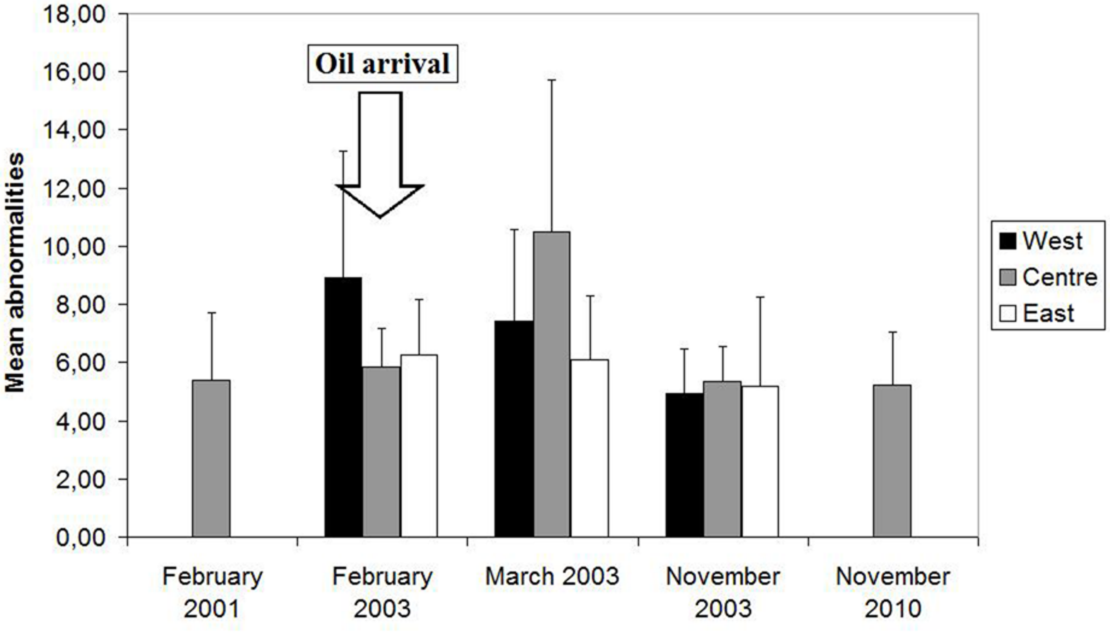

Fig. 2
Mean micronuclei frequency (0/00) and standard deviation (vertical bars) for mussels sampled from West to East in the considered region, in different moments before and after the arrival of petrol from the *Prestige* oil spill.

in this area, which oscillates between 6.25 and 6.08 in the first months going down to 5.17 in November 2003.

3.2 Population diversity at COI sequences

In total 34 different haplotypes of *M. galloprovincialis* were found among the 121 COI sequences obtained, which were submitted to the GenBank (www.ncbi.nlm.nih.gov). They are available under the Accession Numbers JF912338-JF912374.

Significant difference was found for diversity between the West area and the rest (Table 2), western samples exhibiting lower Hd, π and k (z = -1.388 for a Z critical of -1.645 for $p < 0.05$ in a U of Mann-Whitney). However, significant population differentiation (differences for haplotype frequencies between areas) was not found (F_{ST} of 0.00129, 0.00713 and 0.03275 between West-Centre, West-East and Centre-East respectively, all not significant). Differences in haplotype diversity are reflected in the networks constructed from their relationships (Fig. 3). The network obtained from West haplotypes (Figure 3A) had a star-like shape, with a central more frequent haplotype and many singletons derived from it after only one or a few mutations. In contrast, Centre and East networks (Figures 3B and C) exhibited more complex shapes, with internal nodes and various haplotypes of similar relatively high frequency. Estimates of population expansion gave significant results for population expansion in both West and East areas ($p < 0.05$) but not significant for the central region ($p > 0.10$) except for Fu's Fs.

3.3 Proportion of M. edulis introgression

Three pure *M. edulis* genotypes (one in Ortigueira and two in Gijon) and three Glu-5' hybrids (Fig. 4; two from Figueras and one from Toró) were found. These six individuals with *M. edulis* genetic component were distributed across the sampled region: three in the West, two in the centre and one at the East

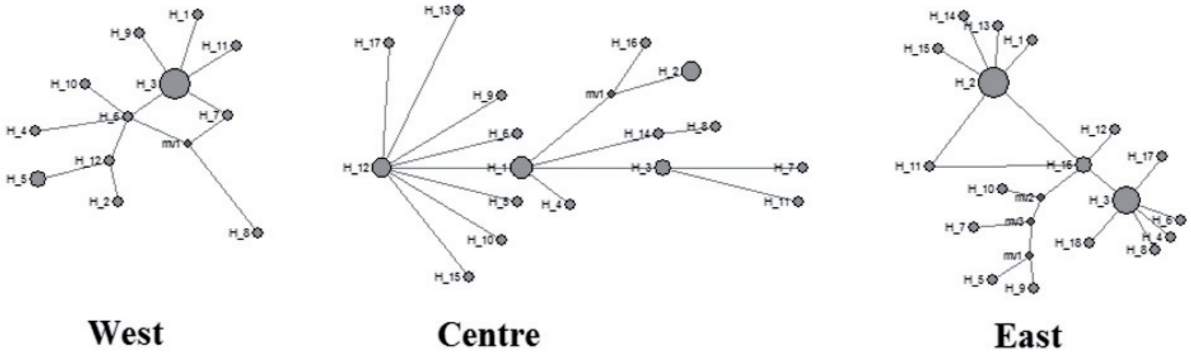

Fig. 3
Haplotype network of *Mytilus galloprovincialis* COI DNA sequences in the West, Centre and East of the studied region.

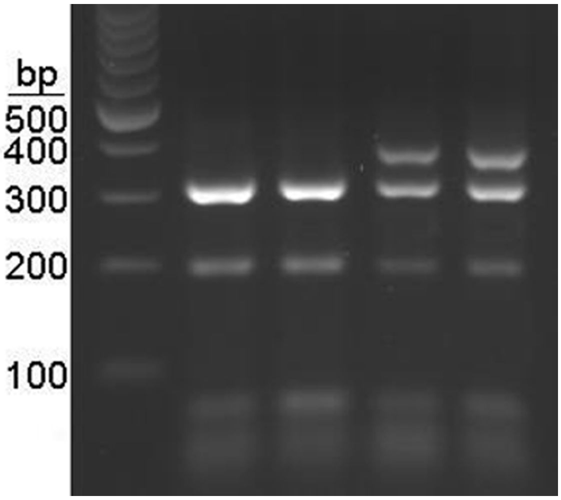

Fig. 4
Photograph of a 2% agarose gel showing the 100bp size marker (lane 1 from the left) and the PCR amplification fragments of pure *M. galloprovincialis* (lanes 2 and 3) and interspecific *M. galloprovincialis* x *M. edulis* hybrids (lanes 4 and 5).

(Table 2). This gives a global proportion of 3.4% individuals with *M. edulis* genetic traces within the *M. galloprovincialis* population of this region. Compared with an average of 9.67% introgression obtained for Atlantic Iberian coasts by Daguin *et al.* (2001), this proportion is statistically lower (z = -2.245, *P* = 0.012). This indicates that the proportion of *M. edulis* variants (alleles and haplotypes) decreased significantly after the *Prestige*.

4. DISCUSSION

The results found in this study reveal a cytological damage after the Prestige oil spill in wild mussels located at the West and the centre of the studied region in the North Iberian Peninsula. This is not absolute cause-effect evidence since control unaffected areas have not been sampled, and temporal changes of basal micronucleus levels may occur in unpolluted sites (Brunetti *et al.* 1992). However, these results are in line with reported DNA damage as a consequence of the accident in Galician mussels (Laffon *et al.* 2006) and strongly suggest that mussels have been also impacted in the neighbouring region. Our results are also hopeful because they demonstrate some recovery. The results obtained after some months were clearly and significantly lower than those after the pollutant arrival (Fig. 2). Return to MN levels similar to those existing pre-oil spill indicates that the mussel population inhabiting North Iberian coasts has overcome the possible cytological damage. Contradictory results have been published about the status of recuperation from cytological damage in nearby eastern regions. One year after the arrival of the fuel oil to the Basque Country, increased MN still occurred in one location (Arrigunaga beach) but not in others (Cajaraville *et al.* 2006). Fuel slicks arrived intermittently to the Basque Country since early summer 2003 (Soriano *et al.* 2006), and, as in the East of the Asturian region here studied, direct association between post-*Prestige* events and MN frequency was not clear in that case. One year was enough for recovery of cytogenetic normality after interrupting urban wastes in Puerto Madryn, Argentina (Machado-Schiaffino *et al.* 2009). In the present study, recovery of normal values one year after the oil spill demonstrates that the recovery of mussels' cytological normality was also rapid.

Persistence of alterations associated with the oil spill has been detected at some levels in different organisms; for example PAH were bioaccumulated in polychaetes (Morales-Caselles *et al.* 2008), fish parasite communities remained altered two years after the oil spill (Perez-del-Olmo *et al.* 2009) and shell morphology changed in *Littorina saxatilis* as well as genetic population pattern for AFLP markers (Piñeira *et al.* 2008). Yamamoto *et al.* (2003) reported that after heavy-oil spill from the Russian tanker *Nakhoda*, it took two to three years for the intertidal organism community to recover to its original level. Similar period time was reported for ecosystem recovery after the *Exxon Valdez* disaster in Alaska (Skalski *et al.*, 2001). In our results, decrease of *Mytilus eduli*s genes in regional *M. galloprovincialis* gene pools suggests a long-term impact on wild mussels. Declines of hybrid genotypes with respect to Iberian pre-*Prestige* values obtained by other authors (Daguin *et al.* 2001) might be due to differential species' sensitivity. *M. edulis* is more sensitive than *M. galloprovincialis* to environmental impacts such as wave exposure, preferring sheltered areas (*e.g.* Bierne *et al.* 2002; Skibinski and Roderick 1991, and references therein).

Another indirect indicator of population disturbance is reduced genetic variation that could have been caused by population reduction. The dramatic cell damage revealed by MN tests is expectedly accompanied by a high mortality rate (*e.g.* Capuzzo *et al.* 1988) and, therefore, reduced population sizes. Initial recovery of genetic diversity is also suggested from our data. Star-shaped haplotype networks are generally considered a signal of recent population expansion after a bottleneck (Mirrol *et al.* 2008), and it could be the case of the West area (Figure 3A), the closest to the accident and more affected by it than the rest of the region. Recovery of normal and homogeneous diversity along the Cantabric coast is expected since mussels constitute a metapopulation (Diz and Presa 2008), also reflected on an absence of spatial differentiation in our results, and individuals coming from less affected areas may replenish depleted subpopulations. However, homogenisation will probably take some generations: differences in diversity among areas can be still detected eight years after the *Prestige* accident.

As a final remark, our results may and hope to send a message. Although Spanish public concern about *Prestige*'s impact decreased in the years following the catastrophe and is negligible today (Fig. 1), long-term impacts of oil spills are unpredictable and may affect the evolutionary path of many species. Prevention is therefore strongly recommended as the best approach. Better containers and maximum control of oil transport operations, as those considerations taken by Europe after the *Prestige* oil spill of banning the use of single-hulled tankers for transporting fuel (Regulation (EC) No 417/2002; http://news.bbc.co.uk/2/hi/europe/2551721.stm), are definitely needed if we want marine ecosystems to be safeguarded in the future.

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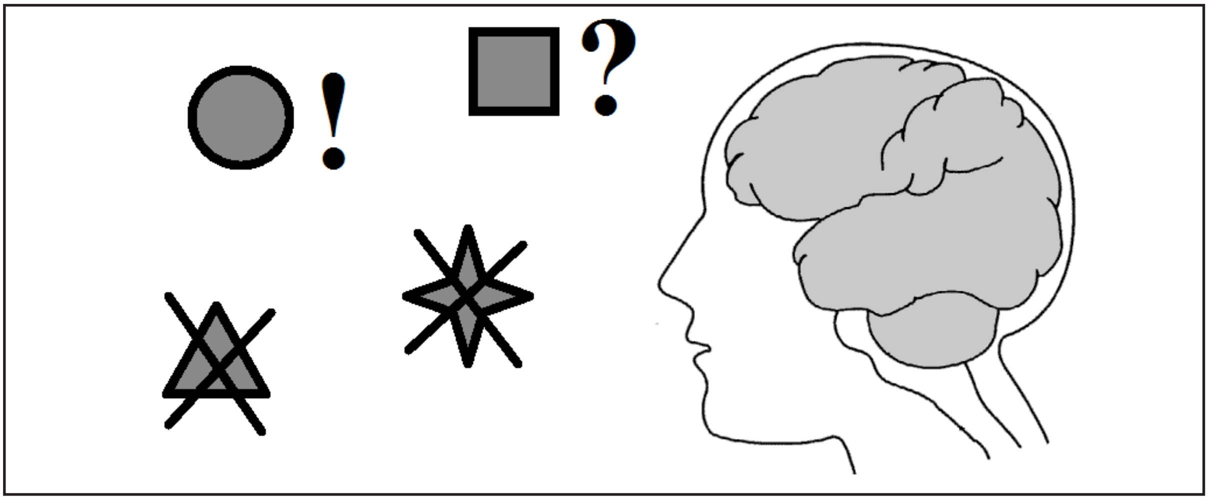
5

DISCUSIÓN



5. DISCUSIÓN

5.1 Discusión de los resultados

Los humanos, conforme fueron aumentando su tamaño poblacional, fueron acentuando su impacto sobre los diversos organismos con los que comparten el hábitat. Los avances en las técnicas de captura y el aumento de la huella ecológica determinan que los efectos antrópicos sean cada vez mayores y hacen imprescindible elaborar normas de obligado cumplimiento para evitar la extinción y mantener el equilibrio en la naturaleza. Pero para la elaboración de las normas es imprescindible tener antes conocimientos profundos de la biología de las especies explotadas, de su entorno general (medio ambiente en el que se encuentran), de la estructura de las poblaciones en cada una de las áreas de explotación y de los efectos que causan las acciones humanas en ellos. En este trabajo se han evaluado tres tipos de impactos con base humana sobre distintas especies marinas como modelos pertenecientes a dos géneros distintos, *Lepidorhombus* y *Mytilus*: efectos de una mala identificación de individuos en pesquerías, efectos derivados de actividades de acuicultura y efectos de un derrame de petróleo. Estos tres factores se incluyen dentro de las ocho grandes amenazas para la naturaleza propuestas por Game *et al.* (2008), confirmando su importancia. Se repasará a continuación la aportación de cada apartado de Resultados a la discusión general sobre la forma en la que las actividades humanas pueden alterar las relaciones entre especies y entre poblaciones dentro de especie en organismos marinos.

El primer artículo analiza el etiquetado de los gallos en rulas y centros comerciales. Se ha observado que se identifica el 90% de los individuos como *L. whiffiagonis* y el 10% como *L. boscii.* Sin embargo, al analizar genéticamente estas muestras con un marcador especie-específico se encuentra que sólo un 49% de los individuos son *L. whiffiagonis* y un 51% son *L. boscii*, concluyendo que en las rulas existe un mal etiquetado de los individuos. Tanto los errores en las rulas como en productos comerciales pueden considerarse accidentales, ya que el precio de ambas especies es el mismo y no hay razones para suponer fraude intencionado. En especies como estos gallos cuya cuota de pesca (TAC) depende de los individuos capturados en la temporada anterior (SeaFish species guide 2011; www.seafish.org), esta mala identificación lleva a estimas erróneas de explotación, lo cual podría a su vez derivar en la sobreexplotación de una de las especies, como fue el caso de las rayas *Dipturus cf. flossada* y *D. cf. intermedia*, cuya mala identificación casi arruina sus pesquerías (Iglésias *et al.*, 2004). Para un manejo sostenible de las pesquerías, la correcta identificación de los individuos es imprescindible para no agravar otros efectos que contribuyan a un descenso del tamaño poblacional y los consiguientes efectos de pérdida de variación genética por deriva y cuellos de botella (Nei *et al.*, 1975; Sbordoni *et al.*, 1986; Fox *et al.*, 2005; Perez *et al.*, 2008).

Por otra parte, puede resultar curioso que se pesquen más *L. boscii* de los esperados. La explicación podría residir en el tamaño relativo de las dos especies, que es menor en *L. boscii*. Existe en los últimos años una reducción continuada del tamaño mínimo permitido de los individuos de *Lepidorhombus* pescados (www.fao.org; www.ices.dk), y la presión de pesca está evidentemente aumentando sobre la especie más pequeña. Un estudio de Audzijonyte *et al.* (2013) advierte que los humanos están cambiando los ecosistemas marinos de todo el mundo directamente a través de la pesca. Los autores demuestran cómo después de 50 años de pesca de un stock, el tamaño corporal medio de los individuos capturados va menguando, y esto puede dar como resultado una disminución en la biomasa y un aumento en el número de capturas del stock (las TAC se calculan en toneladas) de entre un 1 y un 35%. Para los gallos el proceso parece haber empezado, según sugieren los resultados de esta tesis.

En el segundo capítulo se analizan los impactos derivados de granjas de acuicultura sobre las especies nativas del género *Mytilus* en la isla de Vancouver (Columbia Británica, Canadá).

La especie *M. galloprovincialis* es considerada en esta zona como invasora (Braby y Somero, 2006), y lleva cultivándose artificialmente en la isla al menos desde principios del S. XX (Hilbish *et al.*, 2010). La ausencia de barreras hacia el medio natural en dichas granjas favorece que los individuos se escapen e interaccionen con las poblaciones nativas desplazándolas espacialmente al ocupar su nicho ecológico y genéticamente mediante introgresión (Shields *et al.*, 2010). Los resultados obtenidos en este trabajo confirman que los individuos no nativos se han establecido en la naturaleza formando poblaciones locales y generando una zona híbrida (Rawson y Hilbish, 1995; Santaclara *et al.*, 2006). Además, debido a un flujo más o menos continuado de individuos procedentes de granjas, la introgresión de la especie invasora está aumentando (Heath *et al.*, 1995; Shields *et al.*, 2010). Se plantea, a la vista de los resultados, que las zonas híbridas pueden representar vías de expansión para las especies invasoras, siempre que los híbridos posean preferencias ambientales diferentes de la especie invasora pura. En el modelo estudiado, los híbridos entre los invasores y los nativos *M. trossulus* tienen preferencia por hábitats de mar abierto expuestos al oleaje, que no se encuentran entre los hábitats favoritos para *M. galloprovincialis* o *M. trossulus*. Se han descritos invasiones mediadas por caracteres propios de los híbridos en varias especies (Facon *et al.*, 2005; Nolte *et al.*, 2005; Boyer *et al.*, 2008), y según estos resultados podrían incluirse los mejillones del género *Mytilus* entre ellas.

Mallet (2005) considera la hibridación como una invasión del genoma, y comprobó en su revisión que la hibridación se asocia a menudo con alteraciones de hábitat causadas por el hombre (principalmente). La introducción de especies no nativas es una de ellas, ya que nuevos individuos y genotipos pueden ser capaces de desplazar a los ya existentes en una zona mediante introgresión (Arnold, 1992; Martin y Cruzan, 1999; Roques *et al.*, 2001; Sun y Lo, 2011). Más aún, en el caso de mejillones se sabe que la hibridación entre las distintas especies y la formación de híbridos no es un hecho excepcional debido a su estrecha relación evolutiva y por compartir hábitat (Gosling, 1992b; Beaumont *et a*l., 1993; Hilbish *et al*., 2002; Wood *et al*., 2003). Por este motivo, al poner en contacto de manera artificial distintas especies de mejillón mediante las granjas de acuicultura y la liberación de individuos al medio, es todavía mayor la probabilidad de que hibriden con la especie nativa e introduzcan parte de su genoma alterando el acervo genético nativo.

Allendorf *et al*. (2001) muestran cómo las tasas de hibridación e introgresión están aumentando de manera dramática en todo el mundo debido a la introducción de organismos foráneos y a la modificación del hábitat. Los mismos autores explican que una vez ha empezado la hibridación, la introgresión es un proceso casi imposible de parar si los híbridos son fértiles (como es el caso de los mejillones).

El caso considerado en este trabajo es un ejemplo de introducción múltiple y constante de individuos cultivados en poblaciones nativas, lo cual favorece dicha introgresión. Rhymer y Simberloff (1996) advirtieron que la introducción de especies no nativas puede provocar el declive de las poblaciones nativas mediante hibridación e introgresión, un problema que ha sido subestimado por los expertos en conservación y que puede agravarse en el futuro si no se controla adecuadamente la acuicultura.

Ejemplos en especies de peces (Reisenbichler y Rubin, 1999; Utter, 2000) muestran cómo los escapes de granjas están dañando las poblaciones nativas, que pierden su adaptación local y ven así amenazada la viabilidad de las poblaciones, especialmente en circunstancias ambientales adversas (Allendorf *et al*., 2001).

En último lugar, se estudiaron los efectos ocasionados por el vertido del *Prestige* en las poblaciones de los géneros modelo, *Lepidorhombus* y *Mytilus*. En ambos se vio afectado el grado de introgresión entre las especies simpátridas, pero la dirección del cambio fue diferente.

En el género *Lepidorhombus*, se observó un aumento en la tasa de hibridación interespecíficas en el área afectada por el vertido. Las hembras que produjeron la descendencia híbrida analizada fueron *L. boscii* en más del 90% de los casos, lo que sugiere que modificaron su comportamiento permitiendo la fecundación y la hibridación interespecífica. Esto es coherente con los estudios de Martínez-Gómez *et al*. (2006), que muestran una alta sensibilidad de esta especie hacia los residuos tóxicos del *Prestige*.

Los híbridos detectados, que constituían más del 45% de la población en la zona afectada diez años después del vertido, correspondían a generaciones posteriores a la F1, demostrando su fertilidad y sugiriendo la formación de una zona híbrida. Este cambio en un área de distribución de los gallos se correspondió, como era de esperar, con un cambio en la estructura genética poblacional consistente en un aumento de la diferenciación genética entre las poblaciones de zonas afectadas y no afectadas.

Estos resultados también sugieren que este tipo de catástrofes ambientales tienen efectos que van más allá de los encontrados en los primeros momentos, haciendo hincapié en la importancia y necesidad de realizar seguimiento temporal a largo plazo de la evolución de las poblaciones afectadas. Los barcos hundidos, y especialmente los petroleros, arrastran al menos parte de su carga al fondo marino. En el caso que estudia esta tesis el pecio mantiene en sus bodegas cientos de toneladas de fuel, un material peligroso debido a su composición química y su actividad tóxica (Pietrapiana *et al.*, 2002; Thiel, 2003; Laffon *et al.*, 2006; www.cedre.fr/fr/accident/prestige/index.php). Los restos acumulados en las bodegas del *Prestige* se han ido liberando poco a poco debido a la menor densidad de este tipo de fuel y a que no fueron recuperados totalmente (www.cedre.fr). Thiel (2003) sugiere que estos restos son liberados al medio marino contaminando grandes áreas y ayudados por la dispersión de las corrientes marinas, tal como se ve en los efectos persistentes del *Prestige* sobre los gallos y, como se verá más adelante, también sobre los mejillones.

Esto lleva a plantear otra cuestión: ¿puede haber efectos sinérgicos entre los distintos factores que afectan a los gallos? La sinergia es un efecto que se da cuando dos o más factores entran en juego al mismo tiempo de tal manera que el resultado final no es la suma de sus efectos por separado, sino que tienen efectos multiplicativos (Myers, 1995). Por ejemplo, la tolerancia de un individuo a un factor (*v.g.* supervivencia a la toxicidad de un agente contaminante) se vería reducida en presencia de un segundo factor (*v.g.* degradación de hábitat o estrés por pesca). Sin embargo, a pesar de la importancia de las sinergias, apenas se conoce nada sobre su mecanismo y/o influencia debido a la dificultad que supone su estudio. En el caso del género *Lepidorhombus* se han estudiado por separado los posibles efectos de una mala identificación y etiquetado en las pesquerías y los efectos del desastre del *Prestige* a nivel genético. La visión holística de ambos factores podría ayudar a comprender mejor la actual dinámica poblacional de las dos especies de pez gallo. Si tal y como proponen Pauly *et al.* (1998) y Game *et al.* (2009) las actividades marítimas normales (pesca, rutas marinas, etc.) son capaces de alterar ecosistemas y poblaciones, estas alteraciones podrían verse potenciadas por un fenómeno puntual pero cuantitativamente muy importante como el accidente del petrolero *Prestige*. Quizás el aumento relativo de capturas de *L. boscii* sea la causa de una disminución de los stocks de *L. whiffiagonis* por sobrepesca de los mismos. Otra posibilidad es que los híbridos de madre *L. whiffiagonis* sean menos viables que los de madre *L. boscii* y hayan sufrido mayor mortalidad; es conocida la asimetría en las barreras interespecíficas en otros peces (*v.g.* Garcia-Vazquez *et al.*, 2004), y podría suceder también en este género. Con una hibridación tan intensa en las áreas ICES VIIIc y IX como la encontrada en este trabajo, una mortalidad superior en los desoves de *L. whiffiagonis* podría estar también contribuyendo al declive de esta especie, que se reflejaría a su vez en una reducción proporcional de las capturas. Aunque en ausencia de datos sobre los cruzamientos interespecíficos recíprocos esto es solamente una hipótesis, la posibilidad de sinergia entre impactos simultáneos de varios factores hace aún más importante la búsqueda de estrategias de mitigación para estas especies.

En el caso del género *Mytilus* el accidente del *Prestige* tuvo como consecuencia directa una disminución drástica de los tamaños poblacionales de las especies *M. galloprovincialis* y *M. edulis* en el área afectada al dañar tanto a los individuos cómo al hábitat, tal como han publicado numerosos autores (Garcia, 2003; Perez-Cadahía *et al.*, 2004). Antes del *Prestige*, se había descrito una zona híbrida para estas especies localizada entre la costa Atlántica francesa (Figura 12) y las Islas Británicas (Skibinski *et al.*, 1983; Coustau *et al.*, 1991). La zona de la Cornisa Cantábrica y Galicia sería marginal a la zona híbrida, pero aún así se detectaba en ella una introgresión de un 8-11% de genes de *M. edulis* en *M. galloprovincialis* (Daguin *et al.*, 2001).

Si bien una rápida actuación de limpieza ayudó a paliar los efectos de la marea negra, y los estudios citogenéticos de este trabajo muestran una vuelta a valores de normalidad un año después de la catástrofe, se detecta no obstante un impacto a largo plazo a nivel de genética poblacional e introgresión. Ocho años después del vertido, la diversidad genética poblacional era significativamente menor en las áreas más afectadas; y por otra parte, la introgresión de genes de *M. edulis* se había reducido a un 3,41%. La zona híbrida natural entre estas especies parece haber sido reducida tras el *Prestige* y probablemente a causa de él, resaltando la fragilidad de este tipo de áreas ya propuesta por Barton (1979) y Barton y Hewitt (1985).

Es curioso que la especie *M. galloprovincialis* haya prevalecido, al haber disminuido la proporción de genes de *M. edulis*. Esto puede ser indicativo de la resistencia de la primera que, aunque es nativa de la región estudiada, en otros ecosistemas es claramente invasora (McQuaid y Phyllips, 2000; Braby y Somero, 2006), como se comentó anteriormente al tratar los escapes de acuicultura. Las especies con mayor flexibilidad y plasticidad fenotípica suelen convertirse en invasoras más fácilmente cuando se desplazan de su hábitat natural; según los resultados obtenidos en esta tesis a raíz del *Prestige*, *M. galloprovincialis* se adaptaría más fácilmente a condiciones ambientales degradadas o desfavorables, tal como hacen otras especies invasoras (Linde *et al.*, 2008).

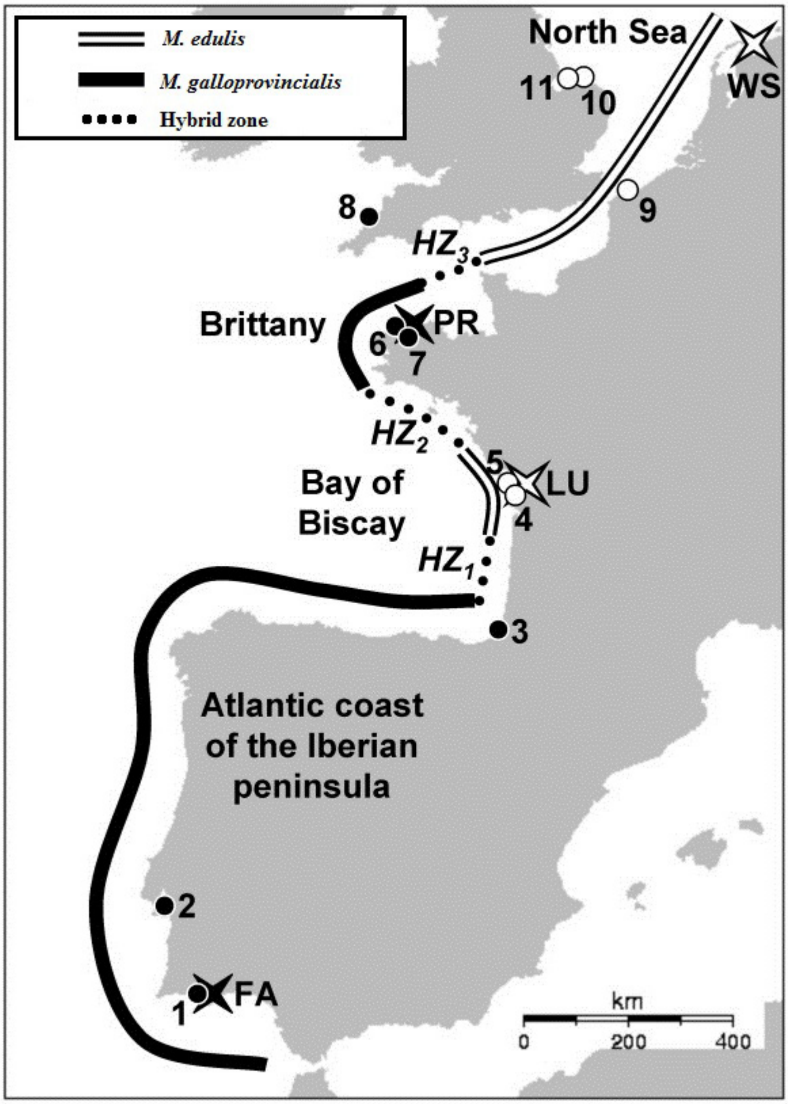


Fig. 12
Distribución de las especies de mejillón en el suroeste europeo, tomado de Faure *et al.*, 2008

5.2 Aplicaciones para la conservación

En esta tesis se ha descrito la aparición de zonas híbridas como consecuencia probable de actividades humanas tan dispares como la acuicultura y los vertidos petrolíferos. Parece que el efecto de la introgresión interespecífica debido a causas antrópicas es común para numerosas especies, y aquí se ha encontrado en modelos vertebrados e invertebrados. Las zonas híbridas naturales se mantienen debido a un equilibrio entre dispersión y selección de los individuos

(Barton y Hewitt, 1985), por lo que no es fácil delimitar tanto las áreas que abarcan como los límites entre las especies implicadas. De cara a la conservación de las especies y el ecosistema, es necesario analizar cada caso por separado ya que, como en los casos presentados en esta tesis, no es lo mismo la hibridación entre especies naturalmente simpátridas que la debida a la introducción de especies alóctonas. Allendorf *et al.* (2001) indican que sería necesario establecer el umbral del porcentaje de hibridación "natural" antes de empezar a intervenir sobre un caso concreto, pero que dicho umbral varía en cada situación y establecer un valor apriorístico sería arbitrario y problemático. Los híbridos pueden suponer un problema para la conservación, pero la hibridación también es un proceso clave en la evolución de las especies (DeMarais *et al*., 1992). Sin embargo, en los casos presentados en esta tesis algunas hibridaciones parecen haber sido provocadas artificialmente y esto podría poner en peligro la supervivencia de las especies implicadas. Parece que lo importante para empezar sería identificar cuántas poblaciones no están aún afectadas, dónde están localizadas y tratar de establecer dónde está el límite de distribución de las mismas (tanto para gallos como para mejillones). Como indica Allendorf *et al.* (2001), cuanto menor sea el número de poblaciones de individuos "puros", más importancia debería darse a la conservación y restauración de las mismas.

En un medio que está sometido a la alteración por parte de diversos efectos antrópicos, la conservación de la diversidad es fundamental debido a su contribución directa al mantenimiento de los ecosistemas y a la productividad de los recursos entre otros factores. Esta diversidad supone un almacén de información genética y un "seguro" frente a eventos inesperados ya que cuantas más variantes haya en una población, más posibilidades tendrá dicha población para sobreponerse a eventos desfavorables (Pearce, 1998; Heal, 2000; Covich *et al*., 2004).

Una manera de preservar las especies, su biodiversidad y proteger los stocks pesqueros sería mediante la creación de Áreas Marinas Protegidas o MPA (Marine Protected Area). La dirección a la que apuntan la mayoría de pesquerías es de posible colapso en los próximos años (revisado en Pauly *et al*., 2002), y aunque estas MPA puedan parecer una solución radical que amenace más aún al sector pesquero, en realidad pueden considerarse como una fuente de individuos y diversidad (Game *et al*., 2009) que contribuyen a aumentar los censos de la especie, también en las áreas colindantes no protegidas. Las especies no son igualmente vulnerables en todo su espectro de distribución, siendo más sensibles las áreas de desove y alimentación, y las rutas de migración (Louzao *et al*., 2006; Shillinger *et al*., 2008). Por tanto, sólo sería necesario proteger áreas clave para cada especie, estableciendo MPA en zonas relativamente pequeñas y delimitadas como una herramienta útil de manejo (NOAA, 2000; Pauly *et al*., 2002; Game *et al*., 2008).

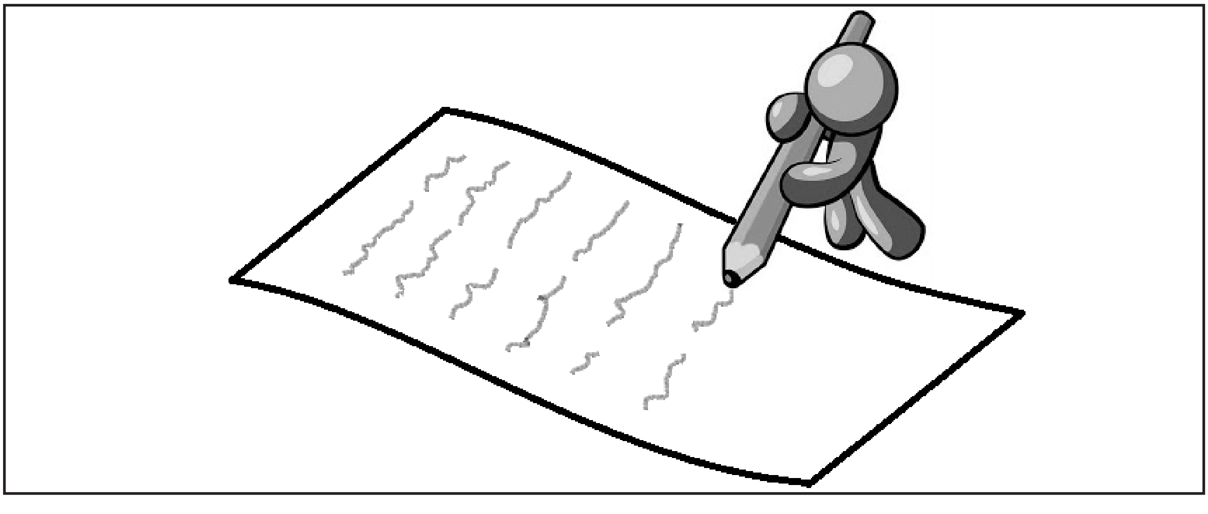
Un ejemplo ilustrativo de la utilidad de un MPA de pequeño tamaño es la definida para el área de alimentación del ave marina *Phoebastria nigripes*, que situada a 4500 Km de su zona de cría ha conseguido reducir substancialmente la mortalidad de esta especie sin necesidad de proteger todo su hábitat (Hyrenbach *et al.*, 2006). También se ha demostrado en diversas especies de peces que la combinación de una MPA y un control de la pesca en el resto de áreas pescables adyacentes ha resultado de gran utilidad a la hora de reconstituir la biomasa de los stocks, ya que el MPA puede proveer de juveniles y adultos a los mismos (Mosquera *et al.*, 2000; Murawski *et al.*, 2000; Roberts *et al.*, 2001).

Por otra parte, la acuicultura supone un problema muy diferente para la conservación. Además de los problemas derivados de los escapes comentados anteriormente, este sistema de producción puede agotar los recursos naturales rápidamente debido a su gran volumen actual (Pullin *et al.*, 1993; Feigon, 2000; Pauly *et al.*, 2002). Bleeker *et al.* (2007) recomiendan un control de las poblaciones cultivadas, así como una mejora de las medidas de seguridad para evitar escapes y un control de las poblaciones nativas para evitar la introgresión génica y su declive. Sayer *et al.* (2011) aportan las sugerencias de eliminar los híbridos y utilizar individuos puros (confirmados genéticamente) y locales para las reintroducciones en el caso de que fueran necesarias. En el caso concreto de las granjas de mejillones de Vancouver es difícil recomendar la primera alternativa, ya que la identificación de los híbridos para su eliminación (se requieren marcadores moleculares) sería una tarea con un presupuesto exagerado. El refuerzo de las poblaciones nativas parecería una buena estrategia. Por otra parte, y dado que las especies invasoras aprovechan los espacios degradados (Linde *et al.*, 2008) o antrópicos (Shields *et al.*, 2008), probablemente la mejora de las condiciones ambientales y la restauración de los hábitats naturales repercutiría en la mejora de las especies nativas.

Como consideración final, el mantenimiento de la biodiversidad es esencial para no agotar los recursos del planeta. Es necesario encontrar estrategias que se sitúen en un punto medio entre los intereses puramente conservacionistas y los las necesidades de explotación de los recursos. En general, ningún método de gestión aislado es lo suficientemente bueno, y por ello es necesario adoptar una mezcla de estrategias, a veces incluyendo algunas que pueden resultar polémicas en un primer momento, pero que a largo plazo llegarán a alcanzar un gran valor, como las áreas marinas protegidas. El análisis de las distintas estrategias y su aplicación para la sostenibilidad en el uso de los recursos naturales ayudará a recuperar y preservar las especies y ecosistemas afectados negativamente por el uso y acción humana.

6

CONCLUSIONES



6 . CONCLUSIONES

6.1 Conclusiones (en español)

1. Empleando marcadores genéticos especie-específicos se ha encontrado un elevado porcentaje de identificación específica errónea en las capturas de gallos (*Lepidorhombus boscii* y *L. whiffiagonis*), tanto en la rula como en los puntos de venta comercial. Esto podría inducir estimas de explotación incorrectas para cada especie y producir sobrepesca de una de ellas, con el consiguiente riesgo de pérdida de tamaño poblacional y diversidad genética.

2. Mediante marcadores genéticos se ha detectado la presencia de zonas híbridas entre especies de mejillones del género *Mytilus* de importación (*M. galloprovincialis*, *M. edulis*) y nativas (*M. trossulus*, *M. californianus*) asociadas a la densidad de instalaciones de acuicultura en la isla de Vancouver. Se describe por primera vez híbridos entre las dos especies nativas, asociados a granjas donde se cultiva una de ellas (*M. trossulus*). Los resultados confirman el impacto genético que puede producir la acuicultura en las poblaciones locales derivado de la introgresión de genomas domésticos en las poblaciones locales. La diferente preferencia de hábitat de los híbridos sugiere que las zonas híbridas pueden ser umbrales para la expansión de las especies exóticas en este género.

3. Las barreras interespecíficas existentes entre las especies del género *Lepidorhombus* parecen haber sido modificadas tras la exposición al fuel vertido por el *Prestige* en las costas españolas, como demuestra una elevada proporción de híbridos detectada mediante marcadores genéticos en el área afectada.

4. Las poblaciones de *Lepidorhombus whiffiagonis* han aumentado significativamente su diferenciación tras el accidente del *Prestige*, debido a la aparición de una zona híbrida en la zona afectada. Las poblaciones de *L. boscii* mantienen sin embargo un nivel similar de diferenciación, que ya era significativa antes del vertido.

5. Las poblaciones costeras de especies simpátridas del género *Mytilus* expuestas al fuel del *Prestige* han recuperado la normalidad citogenética revelada por el test del micronúcleo. En cambio, ocho años después del vertido se comprueba que las poblaciones más afectadas han perdido diversidad genética respecto a las menos afectadas, y que la introgresión de genes de *M. edulis* en *M. galloprovincialis* ha disminuido. Este fenómeno se explica por la mayor resistencia de la segunda especie, que es invasora fuera de su distribución natural, a condiciones ambientales adversas.

6. En las especies modelo empleadas en este trabajo se ha encontrado la aparición de zonas híbridas a consecuencia de alteraciones producidas por actividades humanas en el medio marino. Dada la fragilidad de las zonas híbridas, se propone el fomento de áreas marinas protegidas (MPA) y la recuperación de los hábitats naturales para contribuir a la recuperación y el fortalecimiento de las especies y poblaciones nativas.

6.1 Conclusions (in english)

1. Using species-specific genetic markers we found a high percentage of erroneous identification in megrim catches (*Lepidorhombus boscii* and *L. whiffiagonis*), both in the rula and in supermarkets. This could result in incorrect exploitation estimates for each species and lead to overfishing of one of them, with the risk of loss of both population size and genetic diversity.

2. Using genetic markers we detected the presence of hybrid zones between imported mussels (*M. galloprovincialis*, *M. edulis*) and native ones (*M. trossulus*, *M. californianus*) from genus *Mytilus* associated to the density of aquaculture facilities in Vancouver Island. We describe the first hybrid between the two native species, associated with farms where one of them (*M. trossulus*) is harvested. The results confirm the genetic impact that aquaculture can produce at local populations derived from the introgression of domestic genomes in local populations. The different habitat preference of hybrids suggests that hybrid zones may be thresholds for the expansion of non-native species in this genus.

3. Interspecific barriers existing between the species of the genus *Lepidorhombus* seem to have been modified after exposure to the fuel oil spilled by the *Prestige* off the Spanish coast, as evidenced by a high proportion of hybrids detected in the affected area using genetic markers.

4. The populations of *Lepidorhombus whiffiagonis* have significantly increased their genetic differentiation after the *Prestige* accident due to the appearance of a hybrid zone in the affected area. Nevertheless, the populations of *L. boscii* maintain a similar level of differentiation, which was already statistically significant before the oil spill.

5. Coastal populations of sympatric species of the genus *Mytilus* exposed to the *Prestige* fuel oil recovered normal cytogenetic levels, as revealed by the micronucleus test. However, eight years after the spill we found that the most affected populations have lost genetic diversity in comparison to the less affected ones, and that the introgression of *M. edulis* genes in *M. galloprovincialis* has decreased. This phenomenon is explained by the greater resistance of the second species to adverse environmental conditions, which is invasive outside their natural range.

6. In the model species employed in this study we found the appearance of hybrid zones as a result of changes caused by human activities in the marine environment. Given the fragility of hybrid zones, we propose the development of marine protected areas (MPA) and the recovery of natural habitats to contribute to the recovery and strengthening of the native species and populations.

7

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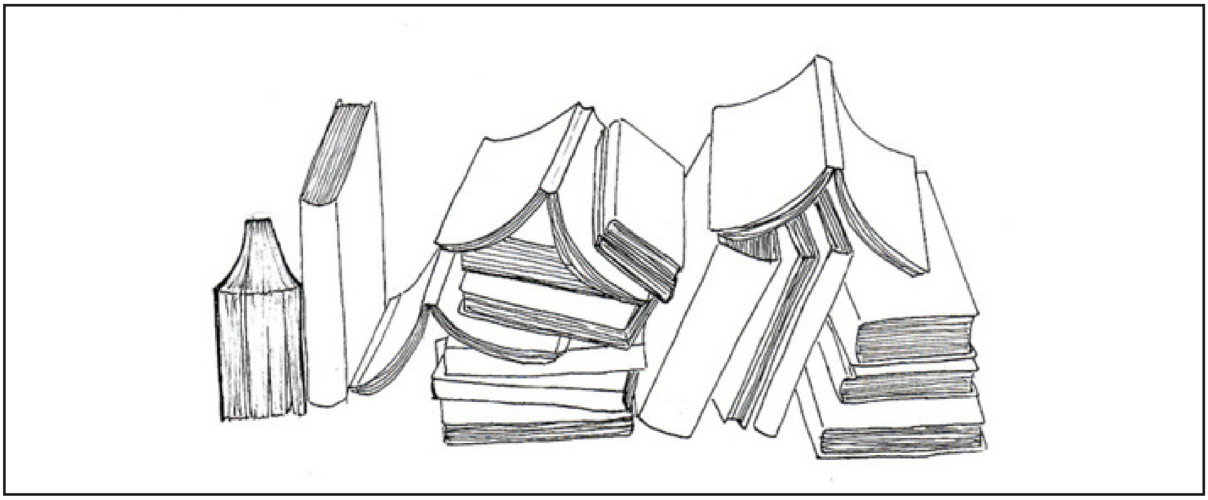

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