UNIVERSIDAD DE MURCIA

PROGRAMA OFICIAL DE DOCTORADO BIOLOGÍA DE PECES: ASPECTOS BÁSICOS Y APLICADOS

FACULTAD DE BIOLOGÍA

DEPARTAMENTO DE ECOLOGÍA E HIDROLOGÍA

"IMPACTO DE LOS RESIDUOS ORGÁNICOS GENERADOS POR LOS CULTIVOS MARINOS EN JAULAS FLOTANTES: ALTERACIÓN DE LA PRODUCCIÓN SECUNDARIA"

(IMPACT OF ORGANIC WASTES GENERATED BY MARINE FISH FARMING IN OPEN SEA NET-CAGES: ALTERATION OF SECONDARY PRODUCCTION)

Memoria que presenta

D. Francisco Navarrete-Mier

para optar al grado de Doctor con Mención Europea por la Universidad de Murcia.

Murcia, septiembre de 2010

UNIVERSIDAD DE
MURCIA

D. Francisco Javier Martínez López, Coordinador del programa de Doctorado "Biología de peces: aspectos básicos y aplicados".

INFORMA:

Que la Tesis Doctoral titulada "Impacto de los residuos orgánicos generados por los cultivos marinos en jaulas flotantes: alteración de la producción secundaria", ha sido realizada por D. Kléber Francisco Navarrete Mier, bajo la inmediata dirección y supervisión de D. Arnaldo Marín Atucha y Carlos Sanz Lázaro, y que el Departamento ha dado su conformidad para que sea presentada ante la Comisión de Doctorado para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 27 de julio de 2010

UNIVERSIDAD DE MURCIA
DEPARTAMENTO DE FISIOLOGÍA
UNIDAD DOCENTE DE
FISIOLOGÍA ANIMAL

Fdo.: Francisco Javier Martínez López
Coordinador del programa de Doctorado

Facultad de Biología
Departamento de Fisiología
Campus Universitario de Espinardo, 30100 Murcia

UNIVERSIDAD DE
MURCIA

D. Arnaldo Marín Atucha, Profesor Titular de la Universidad de Murcia, y Carlos Sanz Lázaro, investigador del grupo de Ecología Acuática de la Universidad de Murcia, AUTORIZAN:

La presentación de la Tesis Doctoral titulada "Impacto de los residuos orgánicos generados por los cultivos marinos en jaulas flotantes: alteración de la producción secundaria", realizada por D. Kléber Francisco Navarrete Mier, bajo nuestra inmediata dirección y supervisión, en el Departamento de Ecología e Hidrología, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 27 de julio de 2010

Fdo.: Arnaldo Marín Atucha

Fdo.: Carlos Sanz Lázaro

Facultad de Biología
Departamento de Ecología e Hidrología
Campus Universitario de Espinardo, 30100 Murcia
T. 868 884 993 – F. 868 884 963 – arnaldo@um.es

UNIVERSIDAD DE MURCIA | Área de Gestión Académica

UNIVERSIDAD DE MURCIA
ÁREA DE GESTIÓN ACADÉMICA
SECCIÓN DE POSTGRADO
Fecha 23-7-2010
SALIDA Nº 350

D. KLÉBER FRANCISCO NAVARRETE MIER
C/ Ángeles, 42, E1, 1 A - Edif. Moreras I
30007 - Casillas
MURCIA

Vista la solicitud presentada el día 29 de junio de 2010 por D. KLÉBER FRANCISCO NAVARRETE MIER, con DNI número X9521235V, sobre autorización de la redacción de tesis doctoral en una lengua distinta al castellano con carácter previo a la tramitación de la misma en la Universidad de Murcia, le comunico que la Comisión de General de Doctorado, vistos

- el informe previo de Ecología e Hidrología, responsable de la autorización de la tesis doctoral en fase de elaboración, de esta Universidad, y
- el visto bueno de la Comisión de Ramas de Conocimiento de Ciencias,

resolvió, en su sesión de 22 de julio de 2010, **ACCEDER** a lo solicitado por el interesado pudiendo, por lo tanto, redactar la tesis doctoral en una lengua distinta del castellano, en inglés, que deberá contener un resumen de la misma en castellano, con una extensión mínima de 2.000 palabras y ser encuadernado como parte de la tesis; el índice y los datos de la portada de la tesis deberán estar en castellano.

Lo que en cumplimiento del artículo 58 de la vigente Ley 30/1992, de Régimen Jurídico de las Administraciones Públicas y del Procedimiento Administrativo Común, de 26 de noviembre, se **notifica a** D. KLÉBER FRANCISCO NAVARRETE MIER, significándole que contra esta resolución, que pone fin a la vía administrativa, se podrá interponer potestativamente ante el mismo órgano que la ha dictado, recurso de reposición, en el plazo de un mes a contar desde el día siguiente a su notificación, de acuerdo con lo dispuesto en el art. 116 de la citada Ley.

Si no hiciera uso del recurso de reposición podrá interponer recurso contencioso-administrativo, en el plazo de dos meses desde la notificación de este acuerdo, en la forma establecida en la Ley 29/1998, de 13 de julio, reguladora de dicha Jurisdicción.

Murcia, 22 de julio de 2010
Vicerrectora de Estudios y
Presidenta de la Comisión General de Doctorado

Concepción Palacios Bernal

UNIVERSITÀ POLITECNICA DELLE MARCHE

DIPARTIMENTO DI SCIENZE DEL MARE

Ancona 28th June 2010

TO WHOM IT MAY CONCERN

Mr. Francisco Navarrete Mier came to work with me at Department of Marine Science , Polytechnic University for three months (March 22 to June 22 of 2010) to improve the knowledge on the impact of fish farm in wild fish/open sea aggregated around culture cages.

Mr Francisco Navarrete Mier turned his attention on the study on fish growth (IGFsystem and myostatin) and animal welfare (Glucocorticoids receptor, Cytokines and HSP70) to establish the effects of net-cages on wild sea bass population and possible gender differences on the sea bass wild population.

During the period of his stay, Mrs. Francisco Navarrete Mier actively participated to all laboratory activities and performed the molecular biology analyses , data treatment, discussion and interpretation of the results. The results Francisco obtained have a particular scientific interest since their originality and two manuscripts are in preparation on the effects at molecular and morphometric levels of net-cages on wild sea bass population

Mr. Francisco Navarrete Mier is a very talented person, particularly accurate in all research activities, and last but not least, has an uncommon capacity to integrate his work within the established research groups.

All these aspects make Mr. Francisco Navarrete Mier a perfect collaborator for any research work .

I can honestly say that it was a pleasure to have him in my laboratory and good came him back to Ancona at any time

I will be happy to confirm what above and/or to provide any further element that you might consider useful for a positive evaluation of his application.

For any other requirement, please do not hesitate to contact me.

Sincerely
Oliana Carnevali

Prof Developmental Biology
Department of Marine Sciences
Università Politecnica delle Marche
Ancona, Italy
Tel.+39 071 2204990
e-mail:o.carnevali@univpm.it

DiSMar

Università Politecnica delle Marche

Dipartimento Scienze del Mare

Ancona 29/09/2010

To Whom it may Concern
University of Murcia

I have read the draft thesis of Francisco Navarrete-Mier. The study evidenced changes of trophic behavior and trophic niche in sea bass *Dicentrarchus labrax* population, associated with fish farm.

This study brings an important contribution for future improvement of the management systems to develop an ecological responsible aquaculture and contribute significantly to the knowledge of the environmental changes in response to the growing anthropogenic activity, such as aquaculture.

The clear experimental design, as well as the techniques used, make this thesis work of high standard quality and therefore should be well-cited. Indeed, the innovative approach used and the dissemination of the data reported on this thesis shall significantly affect the benefit of the end-users as well as of the scientific community.

I have no doubt that the work carried out by D. Francisco Navarrete-Mier is worth of being accepted as fulfillment of the requirements for the award of a PhD

Sincerely

Oliana Carnevali

Prof. Oliana Carnevali
Department of Marine Sciences
UniversitàPolitecnica delle Marche
Via Brecce Bianche
60131 Ancona
ITALY

Tel. +39 071 2204990
Fax. +39 071 2204650

e-mail: o.carnevali@univpm.it

Agradecimientos

Al finalizar esta experiencia tan enriquecedora, como ha sido la realización de esta tesis, quisiera agradecer a todas las personas e instituciones que han sido un apoyo en este camino…

Al programa AlBan (América Latina – Becas de Alto Nivel) por el soporte económico e institucional (Beca No. E07D401236EC).

A Arnaldo confiar en mi incluso sin conocerme, por brindarme la oportunidad de aprender y trabajar junto al grupo. Por la guía científica y por el tiempo dedicado a mi formación. Por darme la libertad de encauzar mi línea investigadora. Por las enseñanzas sobre mar, buceo y navegación. Pero sobre todo por su calidad humana.

A Carlos por brindarme su amistad desde mi llegada y por el tiempo compartido durante las salidas al campo y viajes. Por trabajar con fuerza y alegría, incluso en las tormentas. Y por las clases de estadística, computación, navegación, etc.

A Ma. Dolores a demás del día de tormenta, por impulsarme al trabajo con su permanente dedicación y entrega. Por siempre estar dispuesta al trabajo duro y por llevarme a sus divertidas salidas al campo.

A David C. por la amistad y alegría, compañerismo. Por invitarme siempre a las reuniones, aunque yo no vaya a todas.

A Javi por el ánimo brindado día a día. Por estar siempre dispuesto a explicarme cualquier duda técnica.

A Tano por esa entrega a la ciencia. Por siempre estar dispuesto a explicarme todas mis dudas estadísticas, computacionales y musicales.

A Piedad, Paula, Dani, David S., Oscar, Félix, Dani, Jose, Pedro, Elena, Martha, Simone. El Gran equipo de Ecología Acuática, por compartir día a día las alegrías, frustraciones, proyectos, chistes, artículos y demás, que han hecho que estos tres años a su lado hayan sido una de las mejores experiencias de mi vida.

A Andrés y Pepa V. por también acogerme como parte de su grupo.

A Pepa M. por estar siempre dispuesta con buen humor a cualquier requerimiento en el laboratorio.

A los becarios de Ecología e Hidrología, por el buen ambiente de trabajo.

A Ander por la amistad y por todo el esfuerzo siempre desinteresado que me ha brindado. Por enseñarme sobre la pesca y los peces.

A Pablo por las plumas y todo el soporte técnico.

A Julián por la felicidad de la pesca

Antonio Juan por los análisis en la maravillosa máquina de mercurio.

A Oliana Carnevali, de la Universitá Politecnica delle Marche, por acogerme en su laboratorio, brindarme las facilidades y permitirme realizar una parte importante de este trabajo. Además por los sabios comentarios y su valiosa colaboración.

A Chiara, Beatrice, Giorgia, Ike, Francesca, por estar dispuestos a apoyarme con mis experimentos, aún cuando estaban con mucho trabajo. Grazie mille.

Al personal del Servicio de Cultivos Celulares, Servicio de Biología Molecular y Experimentación Agrícola y Forestal, en especial a Juana, César y Almudena por las facilidades brindadas en el uso de laboratorios y equipos, sin los cuales esta tesis no habría sido posible.

A María Lema del SAI de la Universidade da Coruña por la eficiencia en los análisis.

A Culmarex S.A. por permitirme el ingreso a las granjas y las facilidades brindadas en el desarrollo del trabajo.

A mis familiares y amigos que desde lejos me han acompañado en todo momento.

A mis Padres, Poli y Kléber por estar siempre pendientes de todo. No hay palabras para agradecer todo el esfuerzo.

A Micaela por el cariño y todo lo que nos une.

Finalmente a Patricia, por ser mi compañera y mi motivación a nivel científico y personal. Gracias… totales.

Listado de especies

Nombre científico	Nombre común/ Common name
Boops boops (Linnaeus, 1758)	boga / bogue
Cromis chromis (Linnaeus, 1758)	castañuela / damselfish
Carassius auratus (Linnaeus, 1758)	pez rojo / goldfish
Danio rerio (Hamilton, 1822)	pez cebra / zebrafish
Dicentrarchus labrax (Linnaeus, 1758)	lubina / European seabass
Diplodus puntazzo (Walbaum, 1792)	sargo picudo / sharpsnout seabream
Diplodus sargus (Linnaeus, 1758)	sargo común / White seabream
Diplodus vulgaris (Geoffroy Saint-Hilaire, 1817)	mojarra / common two-banded seabream
Mugil cephalus (Linnaeus, 1758)	mujol / flathead grey mullet
Mytilus galloprovincialis (Lamarck, 1819)	mejillón mediterráneo / common mussel
Oblada melanura (Linnaeus, 1758)	Oblada / saddled bream
Oncorhynchus tshawytscha (Walbaum, 1792)	salmón real / chinook salmon
Ostrea edulis (Linnaeus, 1758)	ostra europea / European flat oyster
Salmo salar (Linnaeus, 1758)	salmón / Atlantic salmon
Sarpa salpa (Linnaeus, 1758)	salpa / salema
Sparus aurata (Linnaeus, 1758)	dorada / gilthead seabream
Symphodus tinca (Linnaeus, 1758)	tordo / east Atlantic peacock wrasse
Thunnus thynnus (Linnaeus, 1758)	atún rojo / bluefin tuna

RESUMEN

Durante el desarrollo de la presente tesis doctoral se han estudiado las posibles alteraciones causadas por los residuos orgánicos generados por los cultivos marinos de peces en jaulas flotantes, enfatizando en los efectos sobre algunos de los productores secundarios asociados. Varios de los impactos en el ambiente provocados por los cultivos en jaulas flotantes han sido previamente estudiados por nuestro grupo de investigación, por lo que con este trabajo se pretende profundizar y ampliar estos conocimientos, para a continuación poder conformar un modelo holístico del sistema.

El trabajo fue realizado en cuatro etapas, a partir de las interrogantes generadas en los estudios previos de nuestro grupo y con desarrollo subsiguiente basado en los resultados obtenidos y la observación *in situ* de las problemáticas existentes. La etapa inicial abordó el estudio de la capacidad de los bivalvos de aprovechar los desechos orgánicos de las granjas de peces, para de esta forma y mediante un policultivo ayudar en la reducción del impacto ambiental generado por las granjas. Demostramos que en mar abierto ésta no es una estrategia efectiva para la mitigación de este impacto, además de considerar que su uso podría causar mayores impactos en el ambiente. En la siguiente etapa se analizó la estructura trófica y los cambios en la acumulación de elementos en la comunidad de biofilm referido a su asociación con las granjas de cultivo de peces. Las comunidades de biofilm muestran una alta capacidad para concentrar elementos provenientes de la actividad de la granja, constituyendo una herramienta fiable en la evaluación del impacto de los residuos disueltos generados por la acuicultura. En la tercera etapa analizamos a nivel de población y de comunidad los peces silvestres asociados a las granjas marinas para determinar su comportamiento trófico relacionado con la presencia de una fuente alimenticia artificial. Los resultados muestran una abundancia de peces notoriamente más alta comparada con zonas no influenciadas por la acuicultura, además esta comunidad de peces silvestres asociados a las granjas presentan una compresión drástica de su nicho trófico, una alteración que podría tener repercusiones severas en los stocks de peces silvestres. Finalmente y basándonos en los hallazgos anteriores, estudiamos las poblaciones de lubina (*Dicentrarchus labrax* L.) que están siendo

influenciadas por la acuicultura, por ser uno de los recursos ícticos más importantes en el Mediterráneo, para ello realizamos análisis a nivel trófico y de expresión génica de moléculas relacionadas con crecimiento, stress y respuesta inmunitaria. Los resultados muestran modificaciones en el nicho trófico y en el perfil de expresión de genes, principalmente relacionados con procesos metabólicos y la actividad fisiológica de los organismos asociados a las granjas. Este trabajo constituye estudio pionero en el análisis del status ecológico, integrando técnicas moleculares e isotópicas para la caracterización de poblaciones de peces en diferentes condiciones ambientales.

Todos estos resultados aportan de manera significativa al conocimiento de las modificaciones ambientales en respuesta a la presencia una creciente actividad antropogénica, como lo es la acuicultura. Asimismo, nos abren nuevas posibilidades de investigación que necesitan ser profundizadas. La valoración integrativa y global de los estudios relacionados, permitirán el desarrollo de sistemas de manejo eficientes que contribuyan al desarrollo de la acuacultura con responsabilidad ecológica.

ABSTRACT

The present thesis involved the study of the organic wastes generated by marine culture of fish floating cages, emphasizing in their effect on the associated secondary producers. Several of the impacts on the environment caused by floating cages fish farms have been previously studied by our research group, in that sense, this work deepen in the understanding of those impacts, and ultimately will allow to figure an holistic modeling of the system.

The work was performed in four stages, starting with questions generated from previous research in the group and subsequently developed in the basis of the results obtained and in the field observation of the existing environmental problems. First, we study the ability of bivalves to exploit organic wastes from the fish farms, with this purpose we test a polyculture system in reducing the environmental impact generated by the farms. We demonstrate that in open sea, integrative culture with bivalves does not mitigate the impact derived from fish culture, thus this is not an appropriate strategy and might even generate other negative impacts on the environment. Next, we analyze the trophic structure and changes in the accumulation of elements in the biofilm community in relation to its association to fish farms. We conclude that biofilm communities show a high capacity to concentrate elements from the farm activity and can therefore be a reliable tool to assess dissolved wastes generated by aquaculture. Subsequently, we analyze at population and community level the wild fish associated to fish farms to determine their trophic behavior in relation to the artificial food source. The results show a higher abundance of fish related to areas not influenced by aquaculture, besides this community of wild fish associated to farms presents a drastic compression of trophic niche. This clear impact could imply a serious impact in the stock of natural wild fish populations. Finally, we analyze the trophic niche and expression of genes related to growth, stress and immune response of European sea bass (*Dicentrarchus* labrax L.) populations being influenced by aquaculture. The results show a modification in trophic niche and in the gene expression profile mainly related to metabolic processes and physiological activity of wild fish associated to fish farms, representing a pioneer work in which the

analysis of ecological status of fish populations integrates isotopic and molecular techniques.

Altogether, these results contribute significantly to the knowledge of the environmental changes in response to the presence of a growing anthropogenic activity, such as aquaculture. Furthermore, this study reveals new research possibilities for integrative and global scientific approach tendencies that allow the development of efficient management systems that contribute to the development of an ecological responsible aquaculture.

ÍNDICE DE CONTENIDOS

CAPÍTULO I: RESUMEN EN CASTELLANO

1 INTRODUCCION

El considerable aumento del consumo de pescado a nivel mundial ha provocado una drástica disminución en los recursos pesqueros, generando una crisis en el sector que ha impulsado un desarrollo global de la acuicultura (CIESM 2007). Durante la última década la industria de cultivo de peces ha crecido a un ritmo medio de 6.5% anual, y se espera que dicha tendencia se mantenga o incremente para las próximas dos décadas (FAO 2006). Esta necesidad de producción ha motivado el desarrollo de sistemas intensivos de acuicultura marina, siendo el sistema de jaulas flotantes uno de los métodos más eficientes, mismo que permite el cultivo de peces en altas densidades y con menores costos de producción ya que elimina la necesidad de recambio de agua y aireación. Esta práctica de cultivo está muy extendida en Europa (Mantzavrakos et al. 2007) teniendo como principales especies objetivo al salmón (*Salmo salar*), la lubina (*Dicentrarchus labrax*), la dorada (*Sparus aurata*) y el atún rojo (*Thunnus thynnus*); aunque la tendencia apunta hacia una mayor diversificación de los cultivos.

Al igual que toda actividad antropogénica, la acuicultura en jaulas flotantes genera un importante impacto en el medio ambiente. La principal fuente de contaminación producida por las granjas flotantes la constituyen los residuos orgánicos liberados a través de las redes en forma de pienso no consumido y de desechos metabólicos de los peces (Focardi, Corsi & Franchi 2005), aunque también pueden afectar otras sustancias derivadas tales como vitaminas, hormonas, metales, biocidas, sustancias anti-incrustantes, etc. (Sanz-Lázaro & Marin 2008). Los desechos generados por las granjas pueden diferenciarse en dos tipos según sus características físicas (1) material particulado con relativamente alto peso que tiende a precipitarse rápidamente, y (2) material particulado lábil o material disuelto, que se incorpora a la columna de agua (Karakassis, Pitta & Krom 2005).

El material particulado de alto peso puede ser consumido por los peces silvestres asociados a las jaulas, sin embargo una gran cantidad se deposita en el bentos a poca distancia de las granjas. La rápida sedimentación de estas partículas en zonas con limitada capacidad de carga, tienden a producir una progresiva disminución del

oxígeno disponible, con el consecuente aumento de las comunidades bacterianas productoras de sulfuros, amonio y metano, las cuales a su vez pueden provocar la muerte de las comunidades bentónicas (Biles et al. 2002; Heilskov, Alperin & Holmer 2006; Holmer et al. 2008).

El material lábil y disuelto se incorpora a la columna de agua y puede dispersarse, con la ayuda de las corrientes, a varios kilómetros de distancia de las granjas. Existen pocos estudios que analicen el efecto de estos materiales disueltos, por lo que no se conoce el impacto que éstos pueden producir, sin embargo se cree que podrían promover cambios en las comunidades de fitoplancton así como afectar a las praderas de pastos marinos (Holby & Hall 1991; Holmer, Perez & Duarte 2003; Sorokin, Sorokin & Ravagnan 1996).

Los policultivos como herramienta para disminuir el impacto ambiental. Uno de los desafíos para el desarrollo de una acuicultura sostenible es la minimización del impacto ambiental que ésta genera. Varios autores han propuesto alternativas como el policultivo de especies con diferentes niveles tróficos, aprovechando así los desechos de una especie para el desarrollo de otra (Jones & Iwama 1991; Neori, Shpigel & Ben-Ezra 2000). En el caso del cultivo de peces en jaulas flotantes, el carbono orgánico en forma de alimento no consumido y de materia fecal producida por los peces, podría representar una fuente disponible de alimento para organismos filtradores como los bivalvos, quienes podrían asimilar parte de este material particulado así como el fitoplancton presente en el área. De esta forma los bivalvos actuarían como filtros biológicos que reducirían el impacto ambiental generado por los cultivos de peces y a la vez incrementarían su crecimiento aumentando así la productividad general de las granjas (Neori, Ragg & Shpigel 1998; Shpigel & Blaylock 1991; Troell et al. 1997).

La lógica de este modelo parece ofrece una alternativa prometedora para alcanzar una acuicultura sostenible. Varios estudios han demostrado su eficiencia en sistemas dulceacuícolas y en sistemas cerrados (Jones & Iwama 1991; Peharda et al. 2007; Troell & Berg 1997); sin embargo en otros sistemas no existen datos concluyentes sobre la efectividad de los mismos (Cheshuk, Purser & Quintana 2003; Stirling & Okumus 1995).

En este sentido, la fase inicial de la presente tesis estudia la eficiencia de dicho modelo en un sistema de aguas abiertas.

Valoración de los residuos disueltos. Los efectos en la generados por las granjas de peces en jaulas flotantes son menos visibles en la columna de agua que en el bentos (Neofitou & Klaoudatos 2008). Las mediciones de ciertos parámetros, suelen estar más influenciadas por la estacionalidad que por los desechos de la acuicultura (Yucel-Gier, Uslu & Bizsel 2008). Así, el monitoreo de la columna de agua es complejo debido a la dificultad de obtener muestras representativas a mediano plazo.

El biofilm es una agregación de organismos heterogéneos que está adheridos unos a otros y/o a una superficie. Esta comunidad está principalmente constituida por bacterias y microalgas que secretan una sustancia polimérica extracelular, esencialmente compuesta por polisacáridos, que facilitan la fijación de la comunidad a cualquier superficie (Characklis & Marshall 2010). El biofilm tiene una capacidad de "memoria" al poder registrar el carbono orgánico disuelto en el agua, así como también los metales y otros nutrientes en suspensión que se adhieren a la capa de exopolímeros (Das et al. 2009). Debido a esto se ha sugerido el uso del biofilm como una herramienta útil en el monitoreo de las turbaciones de origen antropogénico (Morin et al. 2008; Pitta et al. 2006; Pitta et al. 2009). En esta tesis se analiza la capacidad del biofilm de registrar los pulsos de contaminación generados por las granjas en mar abierto.

Los peces silvestres asociados a las granjas. Las estructuras que flotan a la deriva en mar abierto atraen una gran cantidad de peces siendo conocidas como objetos agregadores de peces (FADs) (Castro, Santiago & Santana-Ortega 2001). Las granjas de cultivo actúan como FADs agregando un alto número de peces en sus alrededores (Dempster et al. 2002) que incluso pueden superar en biomasa a los peces cultivados (Sudirman et al. 2009). La razón de esta agregación no está claramente definida, pero no hay duda de que la materia orgánica que se escapa a través de las redes, tiene un efecto altamente atrayente (Freon & Dagorn 2000).

Tampoco está definido si los peces asociados a las granjas y de la misma especie objetivo de cultivo son en realidad peces silvestres que han sido atraídos, sin embargo es muy probable que sean animales escapados del cultivo (Dempster et al. 2007), pero comúnmente llegan a alcanzar tallas muy grandes lo que los convierte en potenciales reproductores. Con el incremento de la maricultura, el riesgo de contaminación genética generado por los peces escapados de cultivo a las poblaciones silvestres es alto, por lo que dicha interacción podría tener como consecuencia, la perdida de la diversidad genética y a la larga conllevaría a la disminución de los stocks silvestres (ICES 2006). Mientras tanto los peces silvestres asociados de especies diferentes a las objetivo del cultivo, encuentran en las granjas una zona protegida en la que se desarrollan y agregan en grandes cantidades (Valle et al. 2007); tal es así que podrían beneficiar a las pesquerías locales, mantener la diversidad íctica o reducir la precipitación al bentos de alimento no consumido, pero su presencia podría traer otros impactos a la zona como lo son la transferencia de enfermedades o el desequilibrio ecológico (Crozier 2000; Sepulveda, Marin & Carvajal 2004). Hasta el momento existen muy pocos estudios que analicen los peces asociados a las jaulas flotantes más allá de su abundancia y diversidad, por lo que un estudio a profundidad es indispensable.

Las poblaciones de lubina (*Dicentrarchus labrax*) asociadas a las granjas. La lubina se encuentra en aguas costeras en la zona noreste del Océano Atlántico desde el sur de Noruega hasta Marruecos además del Mediterráneo y el Mar Negro (Triantafyllidis 2007), constituyendo un recurso muy importante para las pesquerías. Esta especie es además el principal cultivo marino en el sur de Europa (Picchietti et al. 2009), donde es cultivada en granjas flotantes desde hace tres décadas. Siendo posible, incluso, que el número de animales cultivados sea mayor al existente en las poblaciones silvestres (ICES 2006). A demás de estas, existe una población representativa permanentemente asociada a las granjas de cultivo de peces de la que no se conoce con seguridad si son animales silvestres o son peces escapados del cultivo (Dempster et al. 2007). Estos animales asociados alcanzan tamaños superiores a los de las poblaciones costeras, quienes están sometidos a una alta presión pesquera que les impide alcanzar a condiciones ideales de reproducción (Machias et al. 2004). Debido a lo anteriormente expuesto, la lubina es un modelo ideal para investigaciones de interacciones

ecológicas acerca de poblaciones de peces con importancia económica en el Mediterráneo.

2 OBJETIVOS

2.1. Determinar si el cultivo de bivalvos en las proximidades de una granja de peces en mar abierto reduce el impacto ambiental generado por los desechos orgánicos producidos por la granja.

2.2. Evaluar la respuesta del Biofilm bajo la influencia de desechos orgánicos generados por los cultivos de peces en jaulas flotantes la dorada.

2.3. Determinar la modificación de la abundancia y del nicho trófico de las comunidades de peces silvestres asociadas a las granjas de cultivo de peces en mar abierto.

2.4. Evaluar el estatus trófico y la expresión de genes relacionados con el crecimiento, estrés y respuesta inmunitaria en diferentes poblaciones de Lubina (*Dicentrarchus labrax*).

3 PRINCIPALES RESULTADOS Y DISCUSIÓN

3.1 El policultivo con moluscos bivalvos puede reducir el impacto ambiental generado por las granjas marinas de cultivo de peces?

En este trabajo se evaluó la capacidad del cultivo de bivalvos en las proximidades de una granja de cultivo de peces en aguas abiertas, para reducir el impacto generado en el medio ambiente por los desechos orgánicos. El experimento se realizó en los alrededores de una granja cultivo en jaulas flotantes cuyas especies objetivo son dorada (*Sparus aurata*) y Lubina (*Dicentrarchus labrax*) en el mediterráneo oeste. Se colocaron en el agua durante tres meses y a lo largo de un transecto desde 0 a 1800m de distancia de las granjas, dos especies de bivalvos, ostras (*Ostrea edulis*) y mejillón (*Mytilus galloprovincialis*). Se analizó en los bivalvos el crecimiento de las valvas, el peso seco de la carne, la concentración de isótopos estables de carbono y nitrógeno, y la acumulación de metales (Cd, Pb, Cu y Zn). Aún cuando los bivalvos presentaron un crecimiento significativo con respecto a sus tamaños iníciales, la cercanía a la granja de peces no aumenta este crecimiento. Los contenidos de isótopos estables indican que no hay ninguna relación entre la principal fuente de ingreso de materia orgánica generada por la piscifactoría (el alimento balanceado o pienso) y el comportamiento trófico de los bivalvos. Tampoco la acumulación de metales muestra una tendencia a lo largo del gradiente de distancia desde la granja. Los isótopos estables de carbono y nitrógeno así como las concentraciones de metales, demuestran que no hay influencia de los desechos orgánicos generados por las granjas de peces en el crecimiento de bivalvos (*O. edulis* y *M. galloprovincialis*, en este caso). Esto sugiere que los resultados positivos observados en otras áreas geográficas con limitado hidrodinamismo se deben a otras causas indirectas (*p.e.* blooms de plankton). Este trabajo demuestra que el policultivo entre peces y bivalvos no representa una herramienta adecuada para reducir el impacto ambiental de la acuicultura de peces en aguas de mar abierto.

3.2 Respuesta del Biofilm a los desechos de las granjas de cultivo de peces

En este trabajo se analizó estructura trófica y los cambios en la acumulación de elementos en la comunidad de biofilm, debidos al enriquecimiento por materia orgánica, eutrofización, y contaminación por metales derivados de granjas de cultivo de peces. Se cuantificó, en dos estaciones diferentes, la biomasa, el contenido de polisacáridos, el nicho trófico y la acumulación de elementos en el biofilm a lo largo de un gradiente ambiental de desechos de una granja de peces. Se pudo apreciar que la estructura del biofilm y la diversidad trófica fueron influenciadas por la estacionalidad así como por la generación de desechos provenientes de la piscifactoría. La presencia del cultivo de peces aumentó la acumulación de carbono orgánico, nutrientes, selenio y metales en la comunidad de biofilm. El patrón de acumulación de estos elementos fue similar, con independencia de la estructura y el nicho trófico de la comunidad. Las comunidades de biofilm muestran una alta capacidad de concentrar elementos provenientes de la actividad de la granja de peces y que se encuentran disueltos en la columna de agua, además este trabajo demuestra la capacidad del biofilm de "memorizar" eventos esporádicos de ingreso de materia orgánica en el sistema. Esto sugiere que la comunidad de biofilm se puede considerar una herramienta fiable para evaluar los residuos disueltos generados por la acuicultura. Debido a la ubicuidad del biofilm y a su amplia gama de consumidores, su función como sumidero de los residuos disueltos en sistemas costeros, puede tener implicaciones importantes para la transferencia de los desechos de la acuicultura a niveles tróficos superiores.

3.3 Modificación del nicho trófico de los peces silvestres asociados a las granjas de cultivo de peces en mar abierto

Las granjas marinas de cultivo de peces en aguas abiertas funcionan como FADs (dispositivos agregadores de peces) manteniendo altas densidades de peces silvestres permanentemente asociados y que nadan alrededor de las jaulas. En este trabajo se analizó la abundancia de peces y la concentración de isótopos estables de carbono y nitrógeno en peces permanentemente asociados a las granjas marinas en el

Mediterráneo. El análisis se llevó a cabo a nivel de población y de comunidad con el fin de valorar las modificaciones en el comportamiento trófico de los peces ante la presencia de alimento balanceado que se brinda a los peces de cultivo.

La abundancia de peces en los alrededores de la granja fue notoriamente más alta comparada con zonas no influenciadas por la acuicultura marina. La concentración de isótopos estables de carbono y nitrógeno en los peces asociados a las granjas demostró estar directamente influenciada por la pérdida de alimento artificial de la granja a través de las redes. A demás, a nivel de comunidad se aprecia una clara compresión del nicho trófico en los peces asociados, siendo significativamente diferentes a los nichos de las comunidades naturales de peces cuyos nichos son notablemente más amplios. Teniendo en cuenta el elevado número de granjas marinas de peces en el Mediterráneo, el efecto de domesticación en comunidades muy grandes y abundantes de "peces silvestres", podría implicar un serio impacto en el medio ambiente y en particular en los stocks de las poblaciones naturales de peces. Este estudio proporciona una información inicial sobre el estatus trófico de los peces silvestres asociados a las granjas marinas de peces, constituyendo una base para la profundización en estos análisis que a la larga permitirán el desarrollo de estrategias de manejo sobre estos recursos.

3.4 Estatus trófico y expresión de genes relacionados con el crecimiento, stress y sistema inmune en la Lubina (*Dicentrarchus labrax*). Un acercamiento ecológico a las granjas de cultivo de peces en mar abierto

La pérdida constante de alimento de alto nivel proteico a través de las redes de las granjas de cultivo de peces en mar abierto ha provocado un incremento en la condición corporal de los peces asociados a las granjas con respecto a sus pares en zonas sin influencia de la acuicultura. Esta mejor condición corporal puede reflejarse en una mayor tasa reproductiva que contribuiría a las pesquerías locales. La lubina es un pez con muy alta importancia ecológica y comercial, pero en áreas costeras, el incremento de la presión de pesca no permite que se desarrolle hasta alcanzar tallas

óptimas de reproducción. Debido a esto las lubinas que se encuentran permanentemente asociadas a las granjas constituyen un recurso muy importante, sin embargo es probable que una gran cantidad de los individuos silvestres asociados a las granjas, sean en realidad escapes del mismo cultivo. Con el incremento de la maricultura, el riesgo de contaminación génica generado por los escapes es alto. Esta interacción génica podría traducirse en la disminución de los stocks silvestres/ferales de Lubina. El análisis y conocimiento del estado fisiológico individual de las diferentes poblaciones de lubinas permitirá el desarrollo efectivo de estrategias de manejo. En este estudio se han analizado el estatus del nicho trófico y la expresión de genes relacionados con el crecimiento, stress y estado de salud en cuatro poblaciones de dichos peces. Se han analizado peces en diferentes condiciones "naturales" realizando análisis tróficos basados en isótopos estables y análisis de la expresión de genes seleccionados (IGF-I, IGFII, MSTN, GR, HSP70, IL-1β and TNFα). Los resultados muestran que los peces provenientes de cultivo presentan un nicho trófico muy comprimido y con tendencia a fuentes terrígenas, además la expresión de los genes de crecimiento y sistema inmune tienden a un balance cuando se los referencia con peces silvestres. Los peces silvestres presentan el nicho trófico más amplio así como también niveles de expresión de genes relacionados al sistema inmune en bajos niveles y con variaciones mínimas. Además, los peces silvestres asociados a las granjas presentan compresión de nicho similar a los peces cultivados y un nivel intermedio de expresión de IL1-β mientras que la expresión de IGF I, IGF II, MSTN y GR fue muy alta, considerando que estos genes están relacionados con procesos metabólicos, se sugiere que las lubinas asociadas a las granjas tienen una actividad fisiológica alta. Este estudio es, en base a nuestros conocimientos, el primer trabajo que integra caracterizaciones tróficas y moleculares en un análisis ecológico de poblaciones de peces sujetas a diferentes condiciones ambientales y al impacto de la acuicultura.

4 CONCLUSIONES

4.1. Los bivalvos cultivados en las cercanías de granjas de cultivo de peces en mar abierto, no absorben la materia orgánica generada dichas granjas. Por lo tanto el policultivo entre peces y bivalvos no es una medida efectiva para reducir el impacto ambiental que genera la materia orgánica producida por granjas piscícolas en mar abierto.

4.2. La comunidad de biofilm es sensible a la influencia a las granjas marinas de cultivo de peces y responde a esta con cambios estructurales y tróficos. La estacionalidad así como el ingreso de residuos al sistema influyen directamente en la diversidad trófica y en la estructura del biofilm. Debido a esto, se puede considerar a la comunidad de biofilm como una herramienta fiable en la evaluación de residuos disueltos de la acuicultura.

4.3. Los vertidos de materia orgánica provenientes de las granjas de cultivo de peces en jaulas flotantes alteran la abundancia de peces así como el comportamiento trófico de los peces silvestres asociados a la acuicultura, colapsando la amplitud de su nicho trófico.

4.4. La actividad fisiológica de las lubinas asociadas a las granjas de cultivo de peces presentan alteraciones en el comportamiento trófico, así como en la expresión de los genes relacionados con las actividades metabólicas; esto con respecto al estatus presente en poblaciones silvestres o de cultivo.

5 REFERENCIAS

Biles,C.L., Paterson,D.M., Ford,R.B., Solan,M. & Raffaelli,D.G. (2002) Bioturbation, ecosystem functioning and community structure. *Hydrology and Earth System Sciences*, 6**,** 999-1005.

Castro,J.J., Santiago,J.A. & Santana-Ortega,A.T. (2001) A general theory on fish aggregation to floating objects: An alternative to the meeting point hypothesis. *Reviews in Fish Biology and Fisheries*, 11**,** 255-277.

Characklis,W.G. & Marshall,K.C. (2010) *Biofilms: A basis for and interdisciplinary approach*. Wiley-Iterscience, New York.

Cheshuk,B.W., Purser,G.J. & Quintana,R. (2003) Integrated open-water mussel (Mytilus planulatus) and Atlantic salmon (Salmo salar) culture in Tasmania, Australia. *Aquaculture*, 218**,** 357-378.

CIESM. Impact of mariculture on coastal ecosystems. Ciesm Workshop Monographs. Briand, F. www.ciesm.org/online/monographs/lisboa07.pdf 32. 2007.

Crozier,W.W. (2000) Escaped farmed salmon, Salmo salar L., in the Glenarm River, Northern Ireland: genetic status of the wild population 7 years on. *Fisheries Management and Ecology*, 7**,** 437-446.

Das,B.K., Roy,A., Koschorreck,M., Mandal,S.M., Wendt-Potthoff,K. & Bhattacharya,J. (2009) Occurrence and role of algae and fungi in acid mine drainage environment with special reference to metals and sulfate immobilization. *Water Research*, 43**,** 883-894.

Dempster,T., Moe,H., Fredheim,A., Jensen,Q. & Sanchez-Jerez,P. (2007) Escapes of marine fish from sea-cage aquaculture in the Mediterranean Sea: status and prevention. *Impact of mariculture on coastal ecosystems, CIESM Workshop Monographs No. 32* (ed F. Briand), pp. 55-60. Lisboa, 21-24 Feb.

Dempster,T., Sanchez-Jerez,P., Bayle-Sempere,J.T., Gimenez-Casalduero,F. & Valle,C. (2002) Attraction of wild fish to sea-cage fish farms in the south-western Mediterranean Sea: spatial and short-term temporal variability. *Marine Ecology-Progress Series*, 242**,** 237-252.

FAO. The state of World Fisheries and Aquaculture. 2006. Rome, United Nations. Food and Agricultural Organization.

Focardi,S., Corsi,I. & Franchi,E. (2005) Safety issues and sustainable development of European aquaculture: new tools for environmentally sound aquaculture. *Aquaculture International*, 13**,** 3-17.

Freon,P. & Dagorn,L. (2000) Review of fish associative behaviour: toward a generalisation of the meeting point hypothesis. *Reviews in Fish Biology and Fisheries*, 10**,** 183-207.

Heilskov,A.C., Alperin,M. & Holmer,M. (2006) Benthic fauna bio-irrigation effects on nutrient regeneration in fish farm sediments. *Journal of Experimental Marine Biology and Ecology*, 339**,** 204-225.

Holby,O. & Hall,P.O.J. (1991) Chemical Fluxes and Mass Balances in A Marine Fish Cage Farm .2. Phosphorus. *Marine Ecology-Progress Series*, 70**,** 263-272.

Holmer,M., Argyrou,M., Dalsgaard,T., Danovaro,R., Diaz-Almela,E., Carlos,M.D.E., Frederiksen,M., Grau,A., Karakassis,I., Marba,N., Mirto,S., Perez,M., Pusceddu,A. & Tsapakis,M. (2008) Effects of fish farm waste on Posidonia oceanica meadows: Synthesis and provision of monitoring and management tools. *Marine Pollution Bulletin*, 56**,** 1618-1629.

Holmer,M., Perez,M. & Duarte,C.M. (2003) Benthic primary producers - a neglected environmental problem in Mediterranean maricultures? *Marine Pollution Bulletin*, 46**,** 1372-1376.

ICES (2006) *Report of the Working Group on Environmental Interactions of Mariculture (WGEIM)*. Narragansett, Rhode Island, USA.

Jones,T.O. & Iwama,G.K. (1991) Polyculture of the Pacific Oyster, Crassostrea-Gigas (Thunberg), with Chinook Salmon, Oncorhynchus-Tshawytscha. *Aquaculture*, 92**,** 313-322.

Karakassis,I., Pitta,P. & Krom,M.D. (2005) Contribution of fish farming to the nutrient loading of the Mediterranean. *Scientia Marina*, 69**,** 313-321.

Machias,A., Karakassis,I., Labropoulou,M., Somarakis,S., Papadopoulou,K.N. & Papaconstantinou,C. (2004) Changes in wild fish assemblages after the establishment of a fish farming zone in an oligotrophic marine ecosystem. *Estuarine Coastal and Shelf Science*, 60**,** 771-779.

Mantzavrakos,E., Kornaros,M., Lyberatos,G. & Kaspiris,P. (2007) Impacts of a marine fish farm in Argolikos Gulf (Greece) on the water column and the sediment. *Desalination*, 210**,** 110-124.

Morin,S., Duong,T.T., Dabrin,A., Coynel,A., Herlory,O., Baudrimont,M., Delmas,F., Durrieu,G., Schafer,J., Winterton,P., Blanc,G. & Coste,M. (2008) Long-term survey of heavy-metal pollution, biofilm contamination and diatom community structure in the Riou Mort watershed, South-West France. *Environmental Pollution*, 151**,** 532-542.

Neofitou,N. & Klaoudatos,S. (2008) Effect of fish farming on the water column nutrient concentration in a semi-enclosed gulf of the Eastern Mediterranean. *Aquaculture Research*, 39**,** 482-490.

Neori,A., Ragg,N.L.C. & Shpigel,M. (1998) The integrated culture of seaweed, abalone, fish and clams in modular intensive land-based systems: II. Performance and nitrogen partitioning within an abalone (Haliotis tuberculata) and macroalgae culture system. *Aquacultural Engineering*, 17**,** 215-239.

Neori,A., Shpigel,M. & Ben-Ezra,D. (2000) A sustainable integrated system for culture of fish, seaweed and abalone. *Aquaculture*, 186**,** 279-291.

Peharda,M., Zupan,I., Bavcevic,L., Frankic,A. & Klanjscek,T. (2007) Growth and condition index of mussel Mytilus galloprovincialis in experimental integrated aquaculture. *Aquaculture Research*, 38**,** 1714-1720.

Picchietti,S., Fausto,A.M., Randelli,E., Carnevali,O., Taddei,A.R., Buonocore,F., Scapigliati,G. & Abelli,L. (2009) Early treatment with Lactobacillus delbrueckii strain induces an increase in intestinal T-cells and granulocytes and modulates immune-related genes of larval Dicentrarchus labrax (L.). *Fish & Shellfish Immunology*, 26**,** 368-376.

Pitta,P., Apostolaki,E.T., Tsagaraki,T., Tsapakis,M. & Karakassis,I. (2006) Fish farming effects on chemical and microbial variables of the water column: A spatio-temporal study along the Mediterranean Sea. *Hydrobiologia*, 563**,** 99-108.

Pitta,P., Tsapakis,M., Apostolaki,E.T., Tsagaraki,T., Holmer,M. & Karakassis,I. (2009) 'Ghost nutrients' from fish farms are transferred up the food web by phytoplankton grazers. *Marine Ecology-Progress Series*, 374**,** 1-6.

Sanz-Lázaro,C. & Marin,A. (2008) Assessment of Finfish Aquaculture Impact on the benthic Communities in the Mediterranean Sea. *Dinamic Biochemistry, Process Biotechnology and Molecular Biology* 2 (ed Ricardo Russo), pp. 21-32. Global Science Books.

Sepulveda,F., Marin,S.L. & Carvajal,J. (2004) Metazoan parasites in wild fish and farmed salmon from aquaculture sites in southern Chile. *Aquaculture*, 235**,** 89-100.

Shpigel,M. & Blaylock,R.A. (1991) The Pacific Oyster, Crassostrea-Gigas, As A Biological Filter for A Marine Fish Aquaculture Pond. *Aquaculture*, 92**,** 187-197.

Sorokin,Y.I., Sorokin,P.Y. & Ravagnan,G. (1996) On an extremely dense bloom of the dinoflagellate Alexandrium tamarense in lagoons of the Po river delta: Impact on the environment. *Journal of Sea Research*, 35**,** 251-255.

Stirling,H.P. & Okumus,I. (1995) Growth and Production of Mussels (Mytilus-Edulis-L) Suspended at Salmon Cages and Shellfish Farms in 2 Scottish Sea Lochs. *Aquaculture*, 134**,** 193-210.

Sudirman, Halide,H., Jompa,J., Iswahyudin,Z. & McKinnon,A.D. (2009) Wild fish associated with tropical sea cage aquaculture in South Sulawesi, Indonesia. *Aquaculture*, 286**,** 233-239.

Triantafyllidis,A. (2007) Aquaculture escapes: new DNA based monitoring analyses and application on sea bass and sea bream. *Impact of mariculture on coastal ecosystems, CIESM Workshop Monographs No. 32* (ed F. Briand), Lisboa, 21-24 Feb.

Troell,M. & Berg,H. (1997) Cage fish farming in the tropical Lake Kariba, Zimbabwe: Impact and biogeochemical changes in sediment. *Aquaculture Research*, 28**,** 527-544.

Troell,M., Halling,C., Nilsson,A., Buschmann,A.H., Kautsky,N. & Kautsky,L. (1997) Integrated marine cultivation of Gracilaria chilensis (Gracilariales, Rhodophyta) and salmon cages for reduced environmental impact and increased economic output. *Aquaculture*, 156**,** 45-61.

Valle,C., Bayle-Sempere,J.T., Dempster,T., Sanchez-Jerez,P. & Gimenez-Casalduero,F. (2007) Temporal variability of wild fish assemblages associated with a sea-cage fish farm in the south-western Mediterranean Sea. *Estuarine Coastal and Shelf Science*, 72**,** 299-307.

Yucel-Gier,G., Uslu,O. & Bizsel,N. (2008) Effects of marine fish farming on nutrient composition and plankton communities in the Eastern Aegean Sea (Turkey). *Aquaculture Research*, 39**,** 181-194.

CAPÍTULO II: IMPACT OF ORGANIC WASTES GENERATED BY MARINE FISH FARMING IN OPEN SEA NET-CAGES: ALTERATION OF SECONDARY PRODUCCTION

1. DOES BIVALVE MOLLUSC POLYCULTURE REDUCE MARINE FIN FISH FARMING ENVIRONMENTAL IMPACT?

Does bivalve mollusc polyculture reduce marine fin fish farming environmental impact?

Francisco Navarrete-Mier, Department Ecology and Hydrology, University of Murcia. Murcia, 30100, Spain.

Carlos Sanz-Lázaro, Department Ecology and Hydrology, University of Murcia. Murcia, 30100, Spain.

Arnaldo Marín, Department Ecology and Hydrology, University of Murcia. Murcia, 30100, Spain.

Abstract

The ability of bivalve culture in the proximity of an open water fin fish farm to reduce the environmental impact caused by organic wastes was tested. The experiment involved floating net cages containing cultured gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) in the western Mediterranean. Two bivalve species, oyster (*Ostrea edulis*) and mussel (*Mytilus galloprovincialis*), were deployed for 3 months along a distance transect running from 0 to 1800 m from the fish cages. Shell growth, flesh dry weight, the concentration of stable isotopes of carbon and nitrogen, and metal accumulation (Cd, Pb, Cu and Zn) in the bivalves were analyzed. Bivalves showed significant growth compared with their respective starting sizes, although closeness to the fish farm did not enhance such growth. The stable isotopes content indicated that there was no relationship between the main input of organic matter from the fish farm (the feed) and the trophic behavior of the bivalves. Neither did metal accumulation show a trend along the distance gradient from the fish farm. All the results were consistent in indicating that neither oysters nor mussels fed on fin fish farming wastes. This work demonstrates that the polyculture of fin fish and bivalves does not represent an appropriate tool for reducing the environmental impact of fin fish aquaculture in open water.

Keywords: polyculture, bivalve, net-cages, stable isotopes, metals, Mediterranean.

1. Introduction

Intensive open water fish culture is a common method of aquaculture in Europe (Mantzavrakos et al., 2007), particularly in the Mediterranean Sea (Vita et al., 2004), where this activity has sharply increased since 1990 and is expected to rise by 5% annually in the next two decades (FAO, 2006).

Aquaculture has a negative environmental impact due to the release of great amounts of particulate organic matter in the form of suspended detritus (Karakassis et al., 2000; Mazzola and Sarà, 2001), which mainly consists of uneaten feed and excretion products from the cultured fish (Cheshuk et al., 2003; Hall et al., 1992; Holby and Hall, 1991). Most of these wastes are accumulated on the seabed close to the fish farms, causing severe modifications of the physical and chemical characteristics of the benthic environment (Diaz, 2001; Karakassis et al., 2000; Rosenberg et al, 2001; Sanz-Lázaro and Marín, 2008). The result is usually a reduction in the number of species and biodiversity, accompanied by changes in the trophic structure (Otaway, 1995; Sanz-Lázaro and Marín, 2006) and algal blooms in the water column (Angel et al., 2002).

Thus, one of the major challenges for the sustainable development of aquaculture is the minimization of its environmental impact. Several authors have proposed alternatives such as polyculture systems (Jones and Iwama, 1991; Mazzola and Sarà, 2001; Neori et al., 2000). The value of polyculture has been emphasized as an ecological engineering practice to restrain the environmental impact of waste from fish cultivation by recycling particulate and dissolved matter, and to enhance the total farming productivity (Troell et al., 2003).

Organic carbon in the form of uneaten feed and faecal material produced by cultured fish may represent a source of available food for filter-feeding organisms, such as bivalves. Kautsky et al. (1997) proposed an open water integrated culture system whereby filter-feeding bivalves are cultured adjacent to fish floating cages. This system was expected to reduce nutrient loadings by filtering and assimilating particulate wastes (fish feed and faeces) as well as phytoplankton. Some of the waste nutrients released to the local environment would be removed when the cultured bivalves are

harvested. In this way, the bivalves would perform as biological filters and environmental cleaners (Neori et al., 1998, 2000; Shpigel and Blaylock, 1991; Shpigel and Neori, 1996). Thus, the authors concluded that integrated aquaculture could, on the one hand, increase the productivity of bivalve culture and, on the other, reduce fish farm waste loadings and therefore the environmental impact of the same. In the Mediterranean, intensive fish farming could, fro example, be accompanied by the culture of native species of bivalves such as, *Ostrea edulis* and *Mytilus galloprovincialis*.

The concept of an integrated bivalve–fish culture model is attractive and appears to offer promising prospects. A few studies regarding the potential of polyculture in open water have been undertaken, although the conclusions are not unequivocal. Some authors suggest that bivalves could use fish farm wastes as an additional food supply (Mazzola and Sara, 2001; Peharda, et al., 2007), resulting, for example in the increased growth of mussels (Wallace, 1980) and oysters (Jones and Iwama, 1991) cultured adjacent to fish cages. On the other hand, some authors found no significant relationship between the floating fish cages and bivalve growth or bioaccumulation in bivalves (Cheshuk, et al., 2003; Stirling and Okumus, 1995). Hence, the question whether integrated bivalve–fish culture reduces the environmental impact of open water aquaculture remains unresolved.

In recent years, stable isotope analyses have emerged as reliable tools for elucidating trophic structures and inferring pathways of energy flow in food webs (Cifuentes et al., 1988; Fry, 2006). The origin of organic matter deposits in coastal environments has often been elucidated using measurements of $\delta^{13}C$ (carbon) and $\delta^{15}N$ (nitrogen) as stable isotopic composition markers (Fry et al., 1977; Kennedy et al., 2004; Papadimitriou et al., 2005; Sweeney and Kaplan, 1980; Thornton and McManus, 1994). Therefore, the measurement of stable isotopes as markers is one of the most effective methods for ascertaining carbon sources and trophic relationships among organisms (Lojen, et al., 2005; Schwarcz and Schoeninger, 1991; Yoshii et al., 1999), and, in our case, for the analysis of the extent to which fish farm organic wastes are used by bivalves. This method involves a comparison of stable isotope ratios between consumers and the food supply, and depends on differences in isotopic values

between the different food sources (Deegan and Garritt, 1997). Stable isotope analysis has been used with persistent chemicals, such as metals, to relate chemical contamination with trophic community structure (Marín-Guirao et al., 2008). Bivalves are well known bioaccumulators of metals (Daskalakis, K., 1996; Shulkin et al., 2003), a fact that can be used as a complementary tool for assessing fish farm effects, since the diet of cultured fish usually contains a range of trace metals (Dean et al., 2007) and aquaculture also uses compounds containing metals in their activities (net antifouling, paints, fuels, etc). Analysis of elements such as Cu, Cd, Zn and Pb in bivalve tissues would confirm the assimilation of products from fish farming (Watanabe et al., 2008).

The aim of this study was to evaluate the usefulness of the polyculture, involving the bivalves *M. galloprovincialis and O. edulis* in an open water fish farm raising *Dicentrarchus labrax* and *Sparus aurata*, to reduce the environmental impact of the fish farm. To do this, we analyzed different bivalve parameters along an environmental gradient: i) growth ii) stable isotope concentrations and iii) the bioaccumulation of metals (Cu, Cd, Zn, and Pb) due to fish farming.

2. Materials and methods

2.1 *Study area*

The study was conducted in the surroundings of a marine fish farm located in Águilas, SE Spain, (western Mediterranean; 37° 24' 56.2" N, 1° 32' 4.0" W) (Fig. 1), with an average annual production of 1000 tonnes. The farm produces gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) in a 12 net cage system, each cage measuring 25 m in diameter and 19 m in depth. The water temperature at 6 m depth ranges between 21 and 26 °C and the salinity from 37 to 38. The seabed consists of carbonate sand with an unattached coralline algae community, at a mean depth of 31 m.

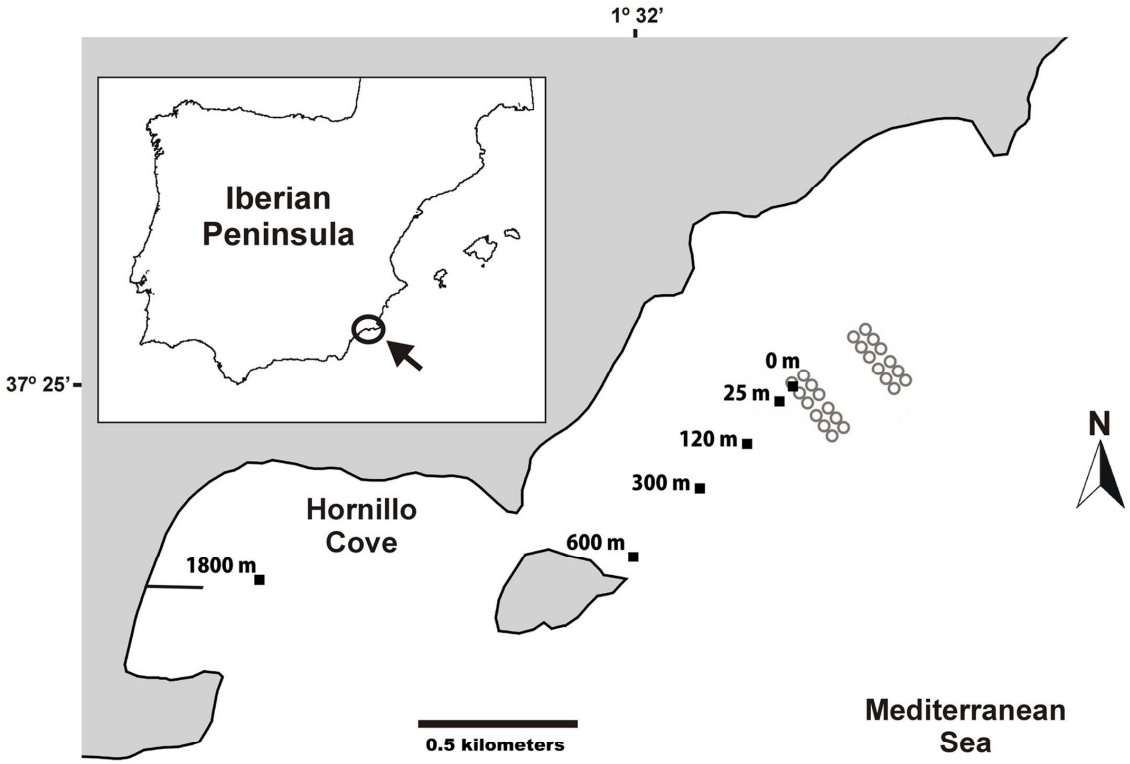

Figure 1. Location of the fish farm and the sampling stations were the bivalves were deployed.

2.2 *Experimental design*

The study was carried out with flat oyster (*Ostrea edulis*, Linnaeus, 1758) and Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) obtained from a commercial shellfish farm located in a non-polluted area close to a marine protected area in Santa Pola (Alicante, Spain).

The bivalves were individually measured and placed in commercial cylindrical culture cages containing trays of 0.40 m diameter and 0.12 m high. Bivalves were tagged and placed individually in eight compartments per cage. All specimens were healthy juveniles with initial shell lengths of 479 ± 58 and 444 ± 47 mm (mean ± SD) for oysters and mussels, respectively. The biometrics measurements were also registered at the end of the experimental time following the methodology described by Helm et al. (2004) and Hwang et al. (2007). A total of 112 individuals per bivalve species were measured individually.

The bivalves were deployed in the field during three months (from March 13 to June 11, 2008). The bivalves were placed 0, 25, 120, 300, 600 and 1800 m from the net cages to assess the influence of the fish farm on their development (Fig. 1). The bivalve cages were maintained at 15 m depth to ensure that the animals were not influenced by re-suspension from the seabed. In the laboratory, the bivalves were individually processed for biometric analysis, which focused on growth, measured as length, and dry weight (Helm et al., 2004; Hwang et al., 2007). The bivalves were dissected to separate valves and eliminate the digestive tract. The remaining tissues were rinsed with distilled water, dried by lyophilization, grounded to a powder and stored at -20 °C until analysis (Riera and Richard, 1996). Samples of the fish feed used in the fish farm during the study period were collected and handled in the same way.

2.3 *Spread and analysis of particulate wastes*

The dispersion of fish farm particulate wastes was studied to ensure that the bivalves were located at distances from the fish farm that were representative of an environmental gradient due to fish farming. To do this, the currents and the sedimentation rates were measured. The currents were measured by means of a current meter (Valeport 106 current meter, Valeport Limited, Dartmouth, UK; located within in the limits of the fish farm next to the fish cages at 15 m depth) during the whole experiment. The sedimentation rates of particulate organic carbon (POC) were measured to analyze the reach and extent of particulate waste dispersion. POC sedimentation rates were obtained by means of sedimentation traps placed along the same spatial gradient as the bivalves at increasing distances from the fish farm (0, 20, 120 and 600 m). The sedimentation traps were made up of four attached cylinders (100 cm height and 12 cm diameter). Each cylinder had a funnel at the bottom, which guided the particulate matter into a 250 ml polyethylene tube, which was too narrow to allow the passage of fish. By means of a mooring system, the sedimentation traps were installed 3 m above the seabed to avoid resuspension (Fig. 2). Five to seven samples, were taken every 5 days, at each station.

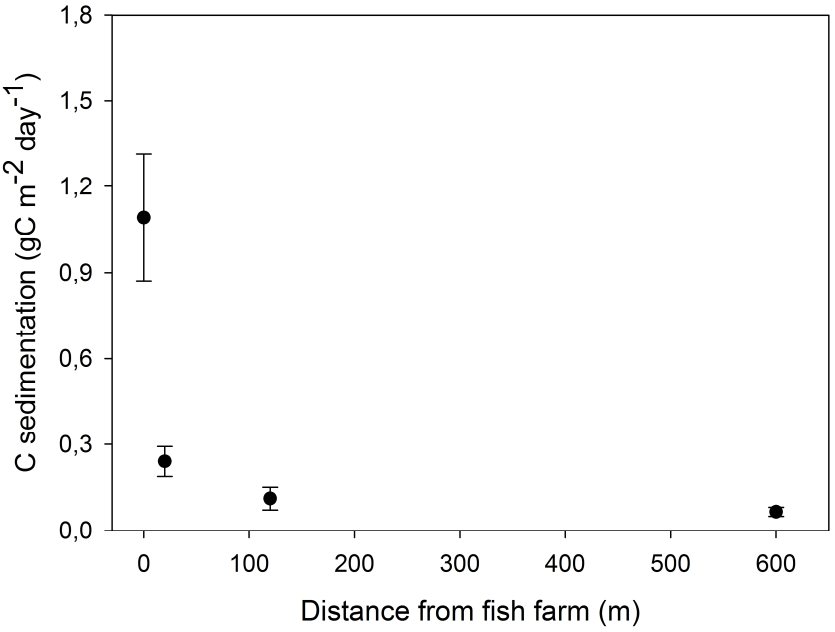

Figure 2. Carbon sedimentation rates (mean ± SE).

2.4 *POC analyses*

The particles collected from the sedimentation traps were dried in an oven (60 °C) to constant weight before and were then finely ground. After a pre-treatment consisting of adding 1:1 HCl, POC was determined using a Carlo Erba Inst. EA 1108 Elemental Analyser (Carlo Erba Strumentazione, Milan, Italy).

2.5 *Stable isotope ratio analyses*

The carbon and nitrogen isotope ratios of the samples were measured with an Flash EA1112 (ThermoFinnigan) elemental analyzer connected to a Delta^{plus} mass spectrometer of isotopic relationships (ThermoFinnigan).

All the isotopic data are reported in the conventional δ notation as follows:

$$\delta^{13}C \text{ or } \delta^{15}N = (R_{sample} / R_{standar} - 1)\ 1000\ (^{o}/_{oo})$$

where *R* represents the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio for δ^{13} C and δ^{15} N, respectively. All $\delta^{13}C$ values were reported as the deviation relative to the Vienna Pee Dee Belemnite

Limestone Standart (v-PDB). The $\delta^{15}N$ standards were calibrated and results are reported relative to atmospheric nitrogen.

2.6 *Metals analyses*

For metal analysis, 0.2 g each of freeze dried mussel flesh, oyster flesh or feed was weighed and placed in a Teflon reactor. After the addition of 3 ml H_2O Milli-Q water, 5 ml concentrated nitric acid (Merk Suprapur), and 2 ml H_2O_2 at 30%; the reactor was maintained in a microwave digester for 20 minutes with a maximum temperature of 210 °C.

Once the sample was cold, the volume was adjusted to 25 ml with Milli-Q water and transferred to a low density polyethylene container. It was then stored at 4 °C until analysis in a spectrophotometer.

Cu, Cd, Pb and Zn were determined by an inductively coupled plasma-mass spectrometer (ICP-MS Algilent 7500 ce, with Octopole reaction system). The detection limits were: Cu, 0.7 µg g^{-1}; Zn, 1.8 µg g^{-1}; Cd, 0.04 µg g^{-1}; Pb, 0.05 µg g^{-1}. The metal concentrations in soft tissues of the bivalves are reported in µg g^{-1} of dry weight (Manaan, 2008).

2.7 *Statistical analyses*

After checking for normality with the Kolmogorov-Smirnov test and homogeneity of variance with the Levene test, one-way analysis of variance (ANOVA) was performed to identify significant differences between stations with regards to growth, stable isotope and metal accumulation in the organisms studied. When significant differences were found between stations, the Bonferroni's test *post hoc* test was performed. To analyse differences between initial and final concentration of metals a one way ANOVA also was used, followed by a Bonferroni´s test post hoc test. In this case comparisons were only performed between the initial value and the value recorded in each

sampling station individually after 90 days. All statistical tests were performed with a significance level of $\alpha = 0.05$.

3. Results

3.1 *Spread and analysis of particulate waste analyses*

The data of the currents showed that the main current was parallel to the coast in both directions (NE and SW). The mean and maximum current speeds along the distance gradient, where the bivalves were deployed (SW direction), were 0.07 and 0.85 m s^{-1}, respectively. So the data concerning the currents confirmed that the cages containing the bivalves were placed along the main current and were thus influenced by the fish farm wastes.

The sedimentation rate of POC was 1.09, 0.24, 0.11 and 0.06 g m^{-2} day^{-1} at 0, 20, 120 and 600 m from the net cages, respectively, following the same distance gradient as that for the studied bivalves (Fig 2). POC sedimentation rates were notably higher below the fish farm than in the rest of the sampling points, where they decreased with distance from the fish farm, following an exponential decay. The behaviour of the POC sedimentation rates along gradient from the fish farm was comparable to that observed in other fish farms (Holmer et al., 2007). These results indicate that the distance gradient where the bivalves were placed for this study was representative of an environmental gradient due to fish farming.

3.2 *Biometric analyses*

The oysters and mussels showed a significant increase in the shell length of 85 ±54 mm and 152 ±45 mm, respectively, between the beginning and the end of the experiment (90 days). The flesh dry weight also decreased by 0.13 ±0.07 g for oysters and 0.13 ±0.04 g for mussels. Neither oysters nor mussels did showed any significant differences in shell length or flesh dry weight (Fig. 3 and Fig. 4) along the gradient.

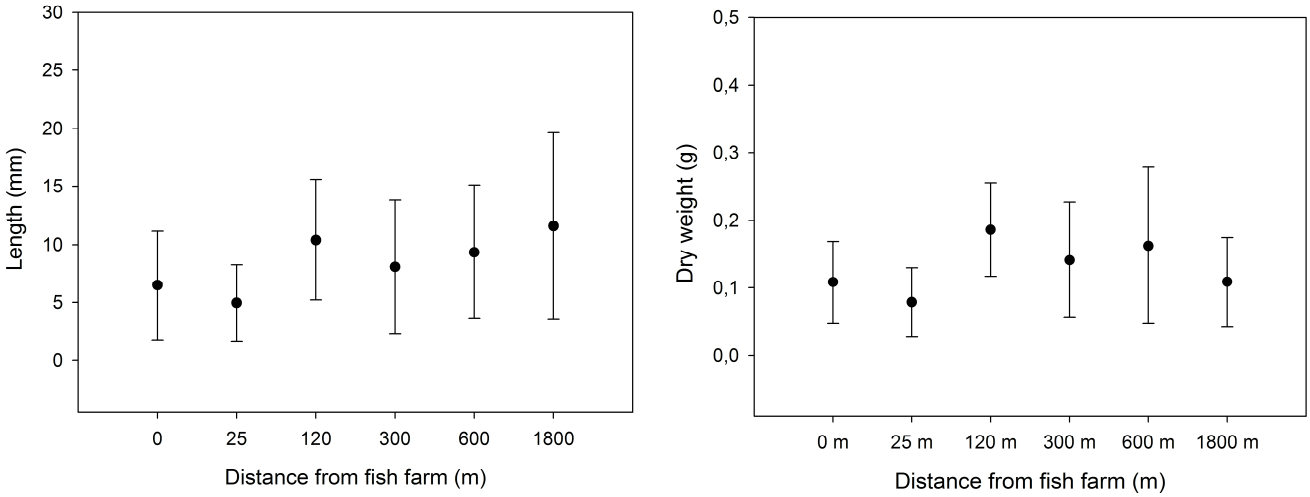

Figure 3. Oyster growth along the distance gradient from the fish farm (mean ± SE, n=12). Growth is shown as the increase in a) length of shell and b) mean dry weight, after 90 days.

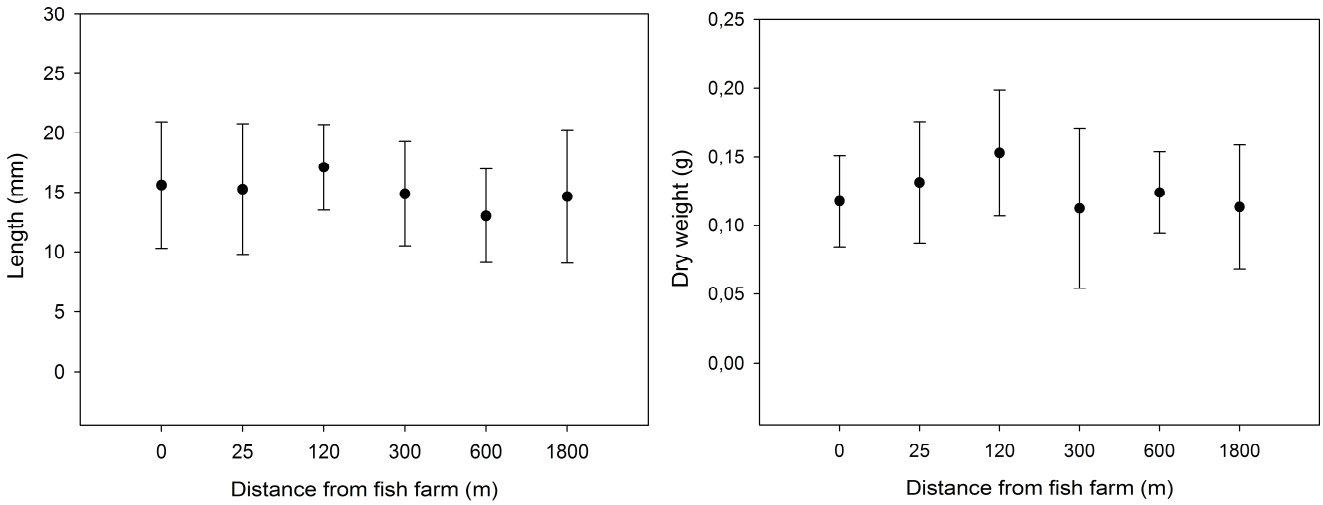

Figure 4. Mussel growth along the distance gradient from the fish farm (mean ± SE, n=12). Growth is shown as the increase in a) length of shell and b) mean dry weight, after 90 days.

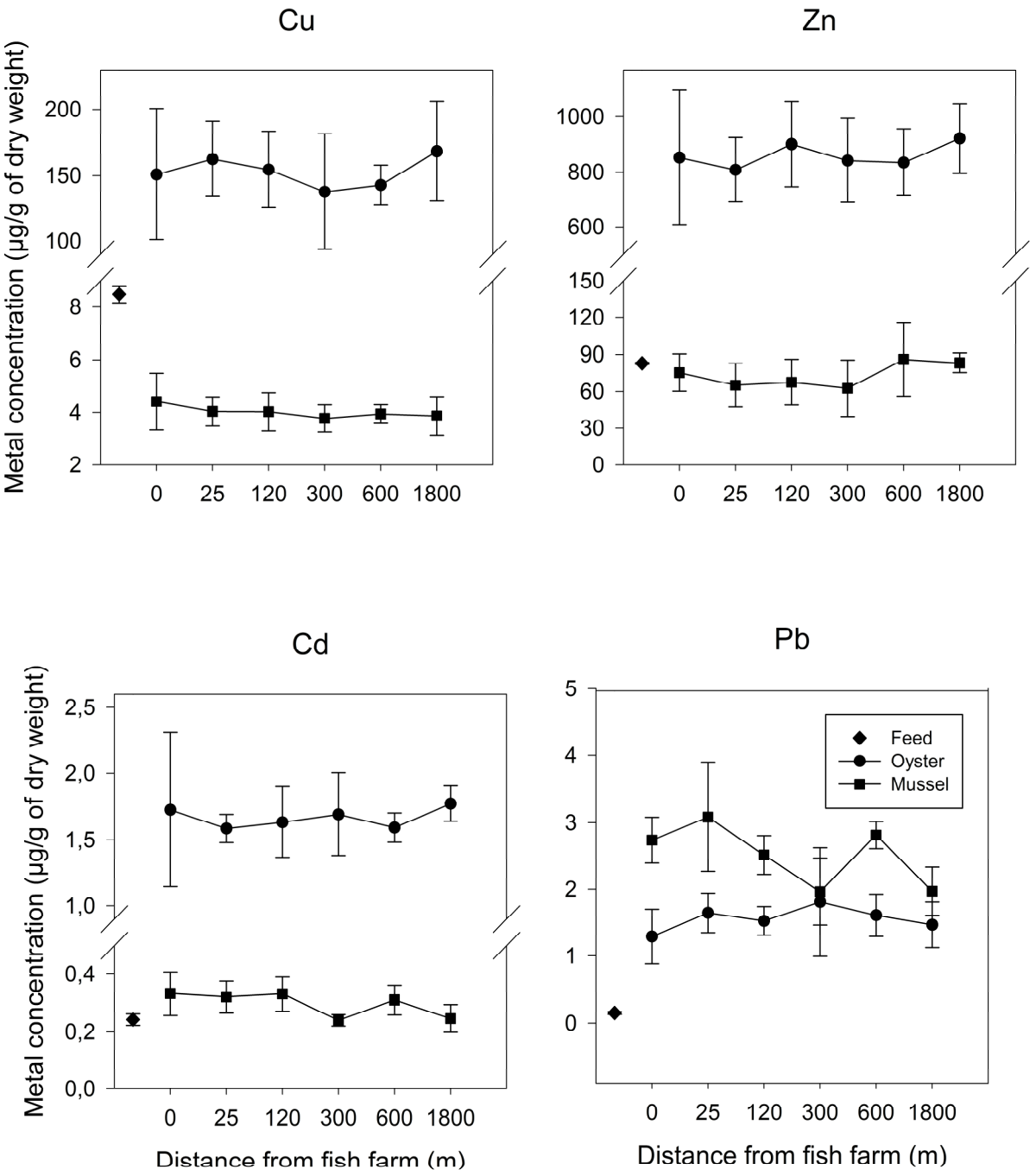

Figure 5. Cu, Zn, Cd and Pb concentrations in oysters, mussels and fish feed after 90 days (mean ± SE, n=4 for bivalves, n=3 for feed).

3.3 *Metal analyses*

The initial metal concentrations in the bivalves were analyzed before the field assays (oysters: Cd= 1.70 ±0.29 µg g^{-1}; Cu= 112.06 ±22.25 µg g^{-1}; Pb= 0.91 ±0.15 µg g^{-1}; Zn= 681.03 ±150.67 µg g^{-1}; mussels: Cd= 0.35 ±0.07 µg g^{-1};Cu= 4.72 ±0.73 µg g^{-1}; Pb= 1.16 ±0.16µg g^{-1}; Zn= 83.92 ±16.59 µg g^{-1}). The metal concentrations determined in the feed were Cd=

0.24 ±0.02 μg g^{-1}; Cu= 8.48 ±0.33 μg g^{-1}; Pb= 0.15 ±0.02 μg g^{-1} Zn= 83.02 ±0.77 μg g^{-1}. For oysters there were no significant differences between the initial and the final metal concentrations at any of the sites. The same was true for mussels, with the exception of the Pb whose initial and final concentrations showed significant differences in most of the sites (Bonferroni´s test, $p<0.05$). Oysters accumulated Cu, Zn and Cd to a greater extent than mussels, but Pb to a lower extent. Metal accumulation was compared between sites, the data pointed to no pattern related with distance from the fish farm and no significant differences between the sites for either oysters or mussels (Fig. 5).

3.4 *Stable isotopic analyses*

The stable isotope composition of the bivalves did not differ significantly between stations. The nitrogen and carbon ratios for oysters at all sites appears in a narrow range ($\delta^{13}C$ = 2.7‰; $\delta^{15}N$ = 0.9‰) (Fig. 6), while for mussels the range was much narrowed ($\delta^{13}C$ = 1.8‰; $\delta^{15}N$ = 1.3‰) (Fig. 7). The $\delta^{13}C$ and $\delta^{15}N$ values for oysters and mussels were very different from the values of the feed, which had a lower $\delta^{13}C$ (-22.3± 0.5‰) and a higher $\delta^{15}N$ (5.3 ± 0.4‰). Moreover, the isotopic composition of both bivalve species was not influenced by the fish farm wastes.

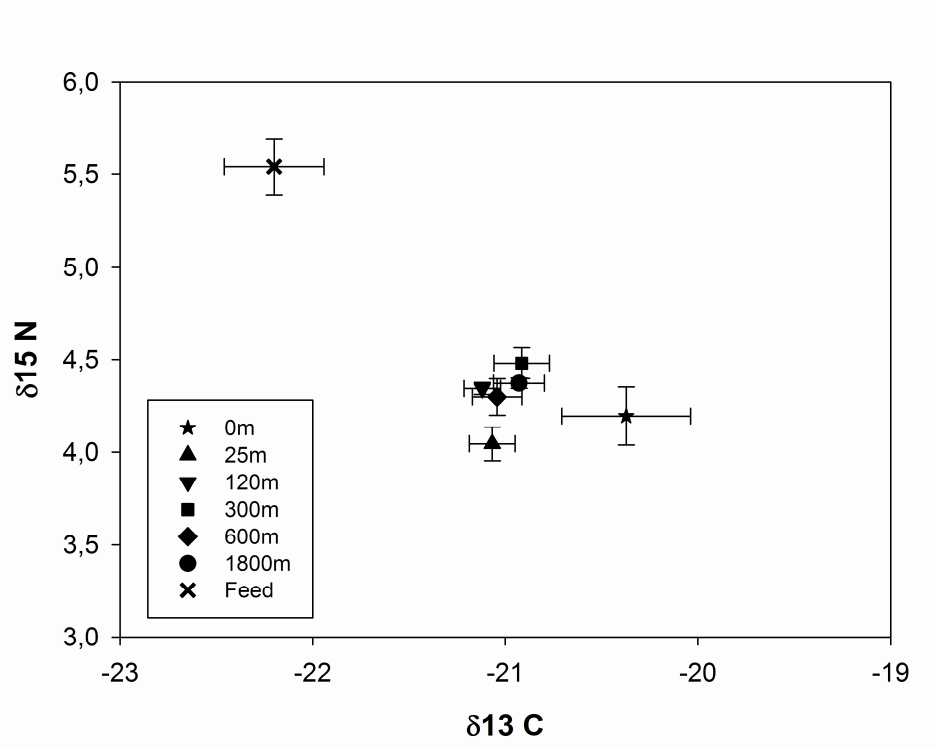

Figure 6. Bi-plot of $\delta^{13}C$ and $\delta^{15}N$ (mean ± SE, n=4 for bivalves, n=3 for feed) of oysters and fish feed after 90 days.

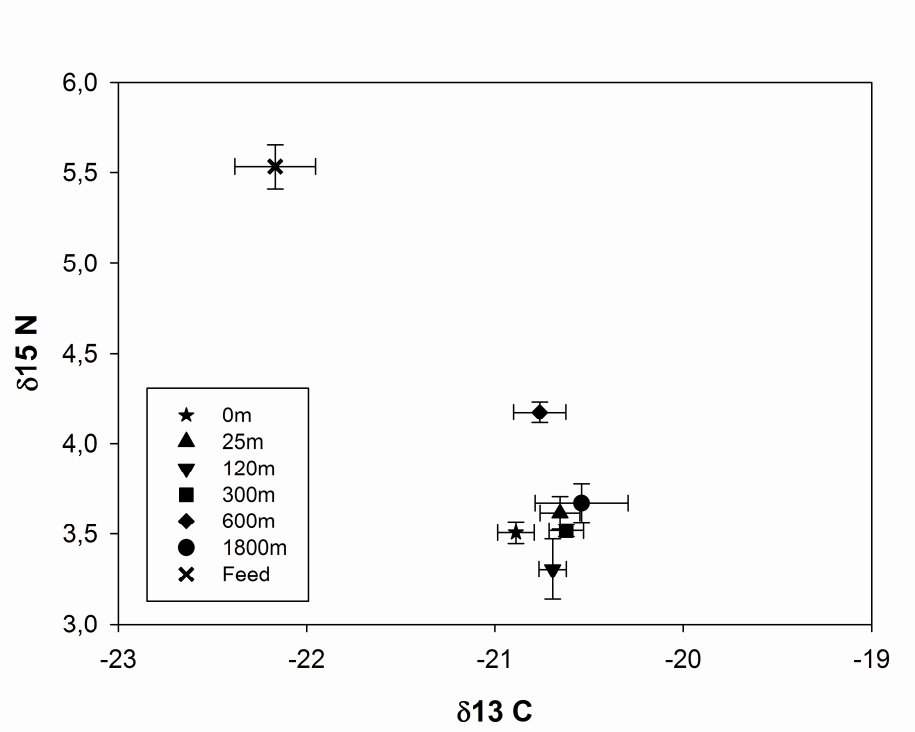

Figure 7. Bi-plot of $\delta^{13}C$ and $\delta^{15}N$ (mean ± SE, n=4 for bivalves, n=3 for feed) of mussels and fish feed after 90 days.

4. Discussion

The present study found no significant influence of fish farm wastes on the growth of *O. edulis* and *M. galloprovincialis*. After three months, the oysters and mussels showed significant growth compared with measurements made at the beginning of the experiment, which indicates good adaptation and an active metabolism (Hwang et al., 2007; Wallace, 1980) (Fig. 3 and Fig. 4). Bivalve growth was similar for all the stations along the field transect, implying that food availability was not greater due to fish farm wastes. Several authors have mentioned that fish farms increase the organic matter in the ecosystem, in general. This, in turn results in an increase in the food availability for filter-feeding organisms and, consequently, an increase in the growth rate of these organism (Cheshuk et al., 2003; Jones and Iwama, 1991; Mazzola and Sarà, 2001; Neori et al., 1998, 2000). Taylor et al. (1992) in *M. edulis* and Cheshuk et al. (2003) in *M. planulatus*, both observed that the distance to fish cages dedicated to *Salmo salar* culture did not influence the growth of these bivalves. Conversely, Jones and Iwama

(1991) observed increased growth in *Crassostrea gigas* near a culture of *Oncorhynchus tshawytscha*. Also, Peharda et al. (2007) found increased growth in *M. galloprovincialis* in a farm of *D. labrax* and *S. aurata*.

As in the case of growth the stable isotope composition of the bivalves did not differ significantly between sites. Stable isotopes are widely used as time-integrating tracers of trophic interactions, as well as to monitor relationships in coastal ecosystems (Lefebvre et al., 2009). Thus, the isotopic analyses showed that bivalves did not change their diet because of the closeness to the farm. Oysters and mussels both had a similar level of $\delta^{13}C$ and $\delta^{15}N$, indicating that the carbon sources and the trophic niche were stable along the transect from the fish farm. Furthermore, isotope levels also indicated that there was no relationship between the main input of organic matter from the fish farm (fish feed) and the trophic behaviour of the bivalves. These results concur with the view that the excess of organic matter exported by the fish farm is not used by the bivalves close the facility. Rather, such enrichment is more likely to make a significant contribution to the production of phytoplankton several kilometres away down current (Danovaro et al., 2004; Holmer et al., 2007).

The assessment of metal accumulation confirmed this view. It has been observed that bivalves in general tend to accumulate metals (Daskalakis, 1996; Shulkin et al., 2003). In sediments around cages, Dean et al. (2007) found that the gradient of metal concentration increased with proximity to the fish farm. Thus, if the organisms used in our experiment were affected by the farm, they should have showed similar behaviour. However, there were no significant differences in the metal concentration of metals, which clearly suggests a different supply of resources for the tested animals. No differences in metal accumulation was found between initial and final values in oysters, and the same being true for mussels with the exception of Pb in most of the stations. These data could imply a greater availability of this metal in the studied area. We found no differences in Pb between the mussels from all the stations, which suggest that Pb enrichment was not due to the impact of the fish farm.

Our results suggest that systems involving the polyculture of bivalves and fish need close analysis before they are implemented. Such polyculture may not only be irrelevant for diminishing the environmental impact derived from fish culture, but they might even generate other impacts, such as a higher load of organic matter from the pseudo-faeces of bivalves, an increased risk of disease to fish and deterioration of the cultivation gear through fouling (Cranford et al., 2009; Hatcher et al., 1994; Mirto et al., 2000). Bivalve integrated coastal aquaculture systems could relieve the eutrophication of coastal water if they are harvested and brought ashore. Although mussels and oysters do not seem to directly incorporate fish farm wastes, the nutrient budget of a water body could benefit from extractive aquaculture (Lindahl et al. 2005) because they feed on phytoplankton, which take up nutrients from the water. However, this profit may only be effective in enclosed water bodies with high nutrient inputs and low seawater exchange.

In conclusion, our study strongly suggests that there is no effect on the growth of bivalves (*O. edulis* and *M. galloprovincialis*, in this case) when subjected to the influence of fish farming. The C and N stable isotope composition and metal concentration demonstrated that these bivalves did not assimilate organic wastes from the studied fish farm, which suggests that the positive effects observed in other geographical areas with limited hydrodynamism were probably due to indirect causes (*e.g.* plankton bloom). Therefore, under open water conditions the integration of bivalves culture in fish farms does not seem to be a useful strategy for diminishing fish farm wastes or for lessening any associated environmental impact.

Acknowledgements

The authors are grateful to the management of Culmarex S.A. who provided farm access and assistance during the field phase of this study. We should also like to thank P. Castillo-Briceño, J. Lloret, M.D. Belando, C. Gutierrez-Cánovas and D. Celdrán for their useful comments. The work was supported by the Programme AlBan, the European Union Programme of High Level Scholarships for Latin America (FNM scholarship No.E07D401236EC) and by FPU predoctoral grant of the Ministerio de Ciencia y Tecnología from Spain.

References

Angel, D. L., Aden, N., Breitstein, S., Yerman, A., Katz, T., Spanier, E., 2002. In situ biofiltration: a means to limit the dispersal of effluents from marine finfish cage aquaculture. Hydrobiología 469, 1-10.

Cheshuk, B.W., Purser, G.J., Quintana, R., 2003. Integrated open-water mussel (*Mytilus planulatus*) and Atlantic salmon (*Salmo salar*) culture in Tasmania, Australia. Aquaculture 218, 357-378.

Cifuentes, L.A., Sharp, J.H., Fogel, M.L., 1988. Stable carbon and nitrogen isotope biogeochemistry in the Delaware estuary. Limnology and Oceanography 33(5), 1102-1115.

Cranford, P. K., Hargrave, B. T., Doucette, L. I. 2009 Benthic organic enrichment from suspended mussel (*Mytilus edulis*) culture in Prince Edward Island, Canada. Aquaculture 292, 189-196.

Danovaro, R., Gambi, C., Luna, G. M., Mirto, S., 2004. Susteinable impact of mussel farming in the Adriatic Sea (Mediterranean Sea): evidence from biochemical, microbial and meiofaunal indicators. Marine Pollution Bulletin 49, 325-333.

Daskalakis, K. D., 1996. Variability of metal concentrations in oyster tissue and Implications to biomonitoring. Marine Pollution Bulletin 32, 794-801.

Dean, R. J., Shimmield, T. M., Black, K. D., 2007. Copper, zinc and cadmium in marine cage fish farm sediments: An extensive survey. Environmental Pollution 145, 84-95.

Deegan, L.A., Garritt, R.H., 1997. Evidence for spatial variability in estuarine food webs. Marine Ecology Progress Series 147, 31-47.

Diaz, R. J., 2001. Overview of Hypoxia around the World. Journal of Environmental Quality 30, 275-281.

FAO., 2006. The state of World Fisheries and Aquaculture 2006. Rome: United Nations, Food and Agricultural Organization.

Fry, B. 2006. Stable isotope ecology. Springer. New York, New York, USA.

Fry, R., Scalan, R. S., Parker, P. L., 1977. Stable carbon isotope evidence for two sources of organic matter in coastal sediments: seagrasses and plankton. Geochimica el Cosmochimica Acta 41, 1875-1877.

Hall, P. O. J., Holby, O., Kollberg, S., Samuelsson, M. O., 1992. Chemical fluxes and mass balances in a marine fish cage farm. IV. Nitrogen. Marine Ecology Progress Series 89, 81-91.

Hatcher, A., Grant, J., Schofield, B., 1994. Effects of suspended mussel culture (*Mytilus* spp.) on sedimentation, benthic respiration and sediment nutrient dynamics in a coastal bay. Marine Ecology Progress Series 115, 219-235.

Helm, M., Bourne, N., Lovatelli, A., 2004. Hatchery culture of bivalves. A practical manual. Roma: Food and Agriculture Organization of the United Nations.

Holby. O., Hall, P. O. J., 1991. Chemical fluxes and mass balances in a marine fish cage farm. II. Phosphorus. Marine Ecology Progress Series 70, 263-272.

Holmer, M., Marba, N., Diaz-Almela, E., Duarte, C.M., Tsapakis, M., Danovaro, R., 2007. Sedimentation of organic matter from fish faros in oligotrophic Mediterranean assessed trough bulk and stable isotope ($\delta^{13}C$ and $\delta^{15}N$) analyses. Aquaculture 262, 268-280.

Hwang, J. J., Yamakawa, T., Aoki, I., 2007. Growth of wild pearl oysters *Pinctata fucata*, *Pinctata margaritifera* and *Pinctata sugillata* (Bivalvia: Pteriidae) in Taiwan. Fisheries Science 73, 132-141.

Jones, T. O., Iwama, G. K., 1991. Polyculture of the Pacific oyster, *Crassostrea gigas* (Thunberg), with chinook salmon *Oncorhynchus tshawytscha*. Aquaculture 92, 313-322.

Karakassis, I., Tsapakis, M., Hatziyanni E., Papadopoulou, K.-N., Plaiti, W., 2000. Impact of cage farming of fish on the seabed in three Mediterranean coastal areas. ICES Journal of marine Science 57, 1462-1471.

Kautsky, N., Troell, M., Folke, C., 1997. Ecological engineering for increased production and environmental improvement in open sea aquaculture, In: Etnier, C., Guterstam, B., (Eds.), Ecological Engineering for Wastewater Treatment, Lewis Publisher. Chelsea, Michigan. Chapter 36, pp. 496.

Kennedy, H., Gacia, E., Kennedy, D. P., Papadimitriou, S., Duarte, C. M., 2004. Organic carbon sources to SE Asian coastal sediments. Estuarine. Coastal and Shelf Science 60, 59-68.

Lefebvre, S., Harma, C., Blin, J.-L., 2009. Trophic typology of coastal ecosystems based on $\delta^{13}C$ and $\delta^{15}N$ ratios in an opportunistic suspension feeder. Marine Ecology Progress Series 390, 27-37.

Lojen, S., Spanier, E., Tsemel, A., Katz, T., Eden, N., Angel, D. L., 2005. $\delta^{15}N$ as a natural tracer of particulate nitrogen effluents released from marine aquaculture. Marine Microbiology 148, 87-96.

Maanan, M., 2008. Heavy metal concentrations in marine molluscs from the Morocan coastal region. Environmental Pollution 153, 176-183.

Mantzavrakos, E., Kornaros, M., Lyberatos, G., Kaspis, P., 2007. Impacts of a marine fish faro in Argolikos Gula (Greece) on the water column and the sediment. Desalination 210, 110-124.

Marín-Guirao, L., Lloret, J., Marin, A., 2008. Carbon and nitrogen stable isotopes and metal concentration in food webs from a mining-impacted coastal lagoon. Science of the Total Environment 393, 118-130.

Mazzola, A., Sarà, G., 2001. The effect of fish farming organic waste on food availability for bivalve molluscs (Gaeta Gulf, Central Tyrrhenian, MED): stable carbon isotopic analysis. Aquaculture 192, 361-379.

Mirto, S., La Rosa, T., Danovaro,R., Mazzola, A., 2000. microbial and meiofaunal response to intensive mussel-farm biodeposition in coastal sediments of the western Mediterranean. Marine Pollution Bulletin 40 (3), 244-252.

Neori, A., Ragg, N. L. L., Shipigel, M., 1998. The integrated culture of seaweed, abalone, fish and clams in modular intensive land-based systems: II. Performance and nitrogen partitioning within an abalone (*Haliotis tuberculata*) and macroalgae culture system. Aquacultural Engineering 17,215-239.

Neori, A., Shpigel, M., Ben-Ezra, D., 2000. A sustainable integrated system for culture of fish, seaweed and abalone. Aquaculture 186, 279-291.

Lindahl, O., Hart, R., Hernroth, B., Kolberg, S., Loo, L-O., Olrog, L., Rehnstam-Holm, A-S., Svensson, J., Svensson, S., Syversen, U. 2005. Improving marine water quality by mussel farming – a profitable solution for Swedish society. Ambio, 34(2),129-136.

Otaway, N. M., 1995. Assesing Impacts of Deepwater Sewage Disposal: A Cause Study from New Souyh Wales, Australia. Marine Pollution Bulletin 31, 347-354.

Papadimitriou, S., Kennedy, H., Kennedy, D. P., Duarte, C. M., Marbá, N., 2005. Sources of organic matter in seagrass-colonized sediments: A stable isotope study of the

silt and clay fraction from *Posidonia oceanica* meadows in the western Mediterranean. Organic Geochemistry 36, 949-961.

Peharda, M., Zupan, I., Bavcevic, L., Frankic, A., Klanjscek, T., 2007. Growth and condition index of mussel *Mytilus galloprovincialis* in experimental integrated aquaculture. Aquaculture Research 38, 1714-1720.

Riera, P., Richard, P., 2006. Isotopic Determination of Food Sources of *Crassostrea gigas* Along a Trophic Gradient in the Estuarine Bay of Marennes-Oléron. Estuarine, Coastal and Shelf Science 42, 347-360.

Rosenberg, R., Nilsson, H. C., Diaz, R. J. 2001. Response of bentic fauna and changing sediment redox profiles over a hypoxic gradient. Estuarine, Coastal and Shelf Science 53, 343-350.

Sanz-Lázaro, C., Marín, A., 2006. Benthic recovery during open sea fish farming abatement in Western Mediterranean, Spain. Marine Environmental Research 62, 347-387.

Sanz-Lázaro, C., Marín, A., 2008. Assessment of finfish aquaculture impact on the benthic communities in the Mediterranean Sea. In: Dynamic Biochemistry, Process Biotechnology and Molecular Biology 2. Ed Global Science Books. (Special Issue I) pp. 21-32.

Schwarcz, H. P., Schoeninger, M., 1991. Stable isotope analyses in human nutritional ecology. Yearbook of Physical Antrhopology 34. 283-321.

Shpigel, M., Blaylock, R. A., 1991. The Pacific oyster, Crassostrea gigas, as a biological filter for a marine fish aquaculture pond. Aquaculture 92, 187-197.

Shpigel, M., Neori, A., 1996. The integrated culture of seaweed, abalone, fish and clams in modular intensive land-based systems: I. Proportions of size and projected revenues. Aquacultural Engineering Vol.15 No 5, 313-326.

Stirling, H. P., Okomus, I. 1995. Growth and production of mussels (*Mytilus edulis* L.) suspended at salmon cages and shellfish farms in two Scottish sea lochs. Aquaculture 134, 193-210

Shulkin, V.M., Presley, B. J., Kavun, V. Ia., 2003. Metal concentrations in mussel *Crenomytilus grayanus* and oyster *Crassostrea gigas* in relation to contamination of ambient sediments. Environment International 29. 493-502.

Sweeney, R. E., Kaplan, I. R., 1980. Natural abundances of ^{15}N as a source indicator for near-shore marine sedimentary and dissolved nitrogen. Marine Chemistry 9, 81-94.

Taylor, B.E., Jamieson, G., Carefoot, T. H., 1992. Mussel culture in British Columbia: the influence of salmon farms on growth of *Mytilus edulis*. Aquaculture 8, 51-66.

Thornton, S., F., McManus, J., 1994. Application of organic carbon and nitrogen stable isotope and C/N ratios as source indicators of organic matter provenance in estuarine systems: Evidence from the Tay Estuary, Scotland. Estuarine, Coastal and Shelf Science 38, 219-233.

Troell, M., Halling, C., Neori, A., Chopin, T., Bushcmann, A. H., Kaustky, N., Yarish, C. 2003. Integrated mariculture: asking the right questions. Aquaculture 226, 69-90.

Vita, R., Marín, A., Madrid, J. A., Jiménez-Brinquis, B., Cesar, A., Marín-Guirao, L., 2004. Effects of wild fishes on waste exportation from a Mediterranean fish farm. Marine Ecology Progress Series 277, 253-261.

Yoshii, K., Melnik, N. G., Timoshkin, O. A., Bondarenko, N. A., Anoshko, P, N., Yoshioka, T., Wada, E., 1999. stable isotope analyses of pelagic food web in Lake Baikal. Limnology and Oceanography 44, 502-511.

Wallace, J., 1980. Growth rates of different populations of the edible mussel, *Mytilus edulis*, in north Norway. Aquaculture 19, 303-311.

2. BIOFILM RESPONSES TO MARINE FISH FARM WASTES

Biofilm responses to marine fish farm wastes

Carlos Sanz-Lázaro, Department Ecology and Hydrology, University of Murcia. Murcia, 30100, Spain.

Francisco Navarrete-Mier, Department Ecology and Hydrology, University of Murcia. Murcia, 30100, Spain.

Arnaldo Marín, Department Ecology and Hydrology, University of Murcia. Murcia, 30100, Spain.

Abstract

The structural, trophic and element accumulation changes in the biofilm community due to organic matter enrichment, eutrophication and metal contamination derived from fish farming was studied. The biofilm biomass, polysaccharide content, trophic niche and element accumulation was quantified along an environmental gradient of fish farm wastes in two seasons. Biofilm structure and trophic diversity was influenced by seasonality as well as by the fish farm waste load. Fish farming enhanced the accumulation of organic carbon, nutrients, selenium and metals by the biofilm community. The accumulation pattern of these elements was similar regardless of the structure and trophic niche of the community. This suggests that the biofilm community can be considered a reliable tool for assessing dissolved aquaculture wastes. Due to the ubiquity of biofilm and its wide range of consumers, its role as a sink of dissolved wastes may have important implications for the transfer of aquaculture wastes to higher trophic levels in coastal systems.

Keywords: metal accumulation, aquaculture dissolved wastes, organic matter enrichment, community trophic niche, peryphiton.

INTRODUCTION

Biofilm is a ubiquitous community since it invariably develops on all solid surfaces exposed to aquatic environments (Allison and Gilbert, 1992; Rao et al., 1997), where it represents most of the natural microbial population (Costerton et al., 1995). Biofilm is an aggregate of heterogeneous organisms that are attached to each other and/or to a surface. This community is principally constituted by bacteria and microalgae which secrete an extracellular polymeric substance matrix, mainly formed of polysaccharides, which facilitates the attachment of the community to any surface (Characklis and Marshall, 1990). The dissolved fraction of the organic carbon constitutes the main source of energy for bacteria and algae in biofilm (Lock and Ford, 1985), and so they also assimilate nutrients and metals in their dissolved form (Das et al., 2009). The exopolymers of biofilm, due to their physical nature, have a great adsorptive capacity and so a great binding affinity for nutrients and metals (Quigley et al., 2002; van Dam et al., 2002; Aldridge et al., 2010), a characteristic that confers biofilm an important accumulation capacity.

Moreover, biofilm communities are the food source of many types of organisms in aquatic systems such as, invertebrates, including rasping grazers, deposit, planktonic and subdeposit feeders, fish and higher vertebrates (Baird and Thistle, 1986; Decho and Moriarty, 1990; Abreu et al., 2007; Kuwae et al., 2008). Thus, biofilm is considered to represent a trophic link between dissolved compounds in the water column and the higher trophic levels of the ecosystem (Hynes, 1970).

In addition, biofilm can contribute substantially to energy flow and nutrient cycling (Battin et al., 2003), especially in the nitrogen cycle (Baldwin et al., 2006), since in aquatic ecosystems, bacteria play an essential role in mineralization and nutrient cycling (Azam et al., 1994; Azam, 1998).

Marine fin fish farming releases substantial amounts of allochthonous organic matter, nutrients and metals to the environment, an effect that can be noticed up to tens or hundreds of metres (Pitta et al., 1998; Karakassis et al., 2000; Morrisey et al., 2000; Pusceddu et al., 2007; Dean et al., 2007). The impact of fin fish aquaculture has been

widely reported in the benthos, where common degradation patterns have been observed (Kalantzi and Karakassis, 2006), since the seabed is able to record possible detrimental effects to the environment over long periods of time (Danovaro, 2003). In the water column however, the effects are less obvious (Neofitou and Klaoudatos, 2008), and any differences in the measured parameters are usually more influenced by seasonality than aquaculture wastes (Pitta et al., 1998; Yucel-Gier et al., 2008). Indeed, water column parameters are often not correlated with the extent of benthic impact. This negligible effect in the pelagic system has been attributed to the important diluting effect of the sea (Pitta et al., 2006), to the rapid grazing of planktonic ciliates (Pitta et al., 2009) or to the importance of heterotrophic over the autotrophic bacteria (Navarro et al., 2008).

Biomonitoring is a more powerful tool for assessing aquatic ecosystem health than physical and chemical analyses (Morin et al., 2008). The biofilm community has been shown to be sensitive to anthropogenic disturbances such as organic matter enrichment, eutrophication and metal pollution (Vis et al., 1998; Admiraal et al., 1999; Ivorra et al., 1999; Barranguet et al., 2002; Khatoon et al., 2007; Morin et al., 2008). Using these communities on artificial surfaces facilitates the direct comparison between sites without confounding environmental and physical variables (Webster and Negri, 2006). The analysis of biofilm enables medium term rather than momentary states of the studied ecosystem (Brummer et al., 2003). Therefore, biofilm could provide the "memory" of disturbance that the water column seems to lack. Fish farming provides an appropriate scenario to study the effects of some of the most common forms of aquatic pollution (organic enrichment, eutrophication and metal pollution) in biofilm communities in open sea environments.

In ecological studies, stable isotope analyses have emerged as reliable tools for elucidating the trophic niche and inferring pathways of energy flow in food webs (Cifuentes et al., 1988). This method involves the comparison of stable isotope ratios between consumers and food supplies (Deegan and Garritt, 1997). While $\delta^{13}C$ allows the carbon source to be differentiated, $\delta^{15}N$ permits the relative trophic position of an organism to be assessed. Thus, as in the case of organisms, $\delta^{13}C$ and $\delta^{15}N$ analyses can

be applied to whole communities (Kuwae et al., 2008; Marin-Guirao et al., 2008), providing information on changes in the trophic niche of the community. Recently, new metrics have arisen which allow ecologists to quantitatively characterize community-wide aspects, providing new perspectives on food web structure, function and dynamics at a community level (Layman et al., 2007). These metrics, which will be detailed in the Materials and Methods section, have already been applied demonstrating changes in niche variation due to a different number of food sources (Darimont et al., 2009).

The aim of this work was to study the structural, trophic and element accumulation changes in the biofilm community due to organic matter enrichment, eutrophication and metal contamination derived from fish farming. To do this, we measured biofilm biomass, polysaccharide content, trophic niche and element accumulation along an environmental gradient of fish farm wastes in two seasons with differing waste load intensities.

MATERIALS AND METHODS

Experimental design

The study was conducted in the surroundings of a marine fish farm located in Águilas, SE Spain, (western Mediterranean; 37° 24' 56.2" N, 1° 32' 4.0" W), which produces gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). The fish farm consisted of two groups of 12 fish cages with an annual production of 1000 tonnes.

The field assays were performed using glass slides as the artificial substrate for the biofilm community to get attached to them. Glass slides were supported by slide holders. The slide holders, in turn, were maintained 3 m below the water surface by an anchoring system and a buoy. Slides were deployed from a fish cage located at the edge of the fish farm facility along a horizontal transect at 0, 20, 60, 120, 350 and 600 m from the fish cage. In all the stations the minimum depth of the seabed was 18 m. Maintaining the same depth at all stations meant that the biofilm community that developed on the glass slides were homogeneously affected by physical factors (e.g.

temperature and irradiance) and avoided resuspension episodes. The main source of the organic matter and pollutants accumulated by the biofilm community were therefore mainly from the dissolved fraction.

Glass slides were deployed in two seasons (June and September) and in each season for 16 days. These seasons were chosen because of the differing amounts of fish feed used and, so different amounts of waste input. Water temperature at the surface were 19-23 °C and 23-24 °C for June and September, respectively. During both surveys the currents had a mean value of 0.05 m s^{-1} and the main direction of the current was NE (Valeport 106 current meter, Valeport Limited, Dartmouth, UK; located in the fish farm next to the fish cage at a depth of 15 m). The average feed supplied to each fish cage was 425 and 689 kg day^{-1} in June and September, respectively. During the September sampling, the slides placed 20 m from the fish farm were not found and so could not be retrieved.

After retrieval of the slides, they were stored frozen at -20°C, and, before each analysis, the biofilm community was scraped off using clean glass slides. The parameters measured were: dry weight biomass, polysaccharide content, the concentration of stable isotopes ($\delta^{13}C$ and $\delta^{15}N$), total organic carbon (TOC), nutrients [total organic nitrogen (TON) and total phosphorous (TP)], selenium (Se) and metals (Fe, Mn, Cu, Zn, Cd, Pb, Ni, As, Cr, Tl).

During the first survey water samples were taken at each sampling station and TP, Se, Fe, Mn, Cu, Zn, Cd, Pb, Ni, As, Cr and Tl, were analyzed to compare the accumulation capacity of biofilm compared to the concentration in the water column. Water samples were filtered (0.45 µm GF/C Whatman filter) and stored frozen at -20°C prior to analysis. The main input of contaminants in the studied aquaculture system is feed pellets, which were analyzed in the same way as biofilm samples.

Biofilm structure

Biofilm structure was analyzed by quatifying the biomass and polysaccharide content. In order to calculate biofilm community biomass, samples were dried at 60°C until

constant weight. Because the extracellular polymeric substances are composed of polysaccharides (Smith and Underwood, 1998; Stal, 2003), the polysaccharide content was measured using the modified phenol-sulfuric acid method (Pacepavicius et al., 1997).

Biofilm trophic niche

For TOC, TON and the stable isotope concentrations of $\delta^{13}C$ and $\delta^{15}N$, samples were previously freeze dried and ground. The carbon and nitrogen isotope ratios of the samples were measured with an elemental analyzer Flash EA1112 (ThermoFinnigan) connected with a mass spectrometer of isotopic relationships Delta^{plus} (ThermoFinnigan).

All the isotopic data are reported in the conventional δ notation as follows:

$$\delta^{13}C \text{ or } \delta^{15}N = (R_{sample} / R_{standar} - 1)\ 1000\ (^{o}/_{oo})$$

where *R* represents the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio for δ^{13} C and δ^{15} N, respectively. All $\delta^{13}C$ values were reported as the deviation relative to the Vienna Pee Dee Belemnite Limestone Standart (v-PDB). The $\delta^{15}N$ standards were calibrated and results were reported relative to atmospheric nitrogen.

Biofilm elemental analysis

For TP, Se, Fe, Al, Mn, Cu, Zn, Cd, Pb, Ni, As, Cr and Tl analysis, samples were freeze dried and then ground. Afterwards, 0.2 g of sample was weighed and placed in a Teflon reactor. After the addition of 3 ml ultrapure water, 5 ml of concentrated HNO_3 (Merk, Suprapur) and 2 ml of 30% H_2O_2 (Merk, Suprapur), the reactor was maintained in a microwave digester for 20 minutes at a maximum temperature of 210 °C. Following the acid digestion, the content of each vessel was poured into volumetric flasks and ultrapure water was added to make up the final volume to 25 ml. Then samples were stored at 4 °C until quantification. The mentioned elements were determined by an inductively coupled plasma-mass spectrometer (ICP-MS Algilent 7500 ce, with Octopole reaction system). The detection limits of the ICP-MS (calculated as three

times the standard deviation of the blanks) were sufficiently low to analyse the sample concentrations. Element recovery was verified using certified reference material (Lagarosiphon major, CRM 60; Community Bureau of Reference, Commission of the European Communities).

Data analysis

Pearson correlation analysis was performed between the biofilm polysaccharide content of June and September, and between the polysaccharide content and biomass of biofilm in each season independently. If data did not meet parametric assumptions, a Spearman correlation analysis was used.

A two-way ANOVA was performed for the biofilm biomass and polysaccharide, TOC, TON, TP, $\delta^{13}C$ and $\delta^{15}N$ content to detect significant differences between treatments in each factor (season and distance from the fish farm) and the interactions between the two factors. When significant differences were found, the Bonferroni post-hoc test was performed. To compare the trend of the measured parameters along the transect from the fish farm between both seasons, the slope of the regressions was compared by the method described in Zar (, 1984), which is equivalent to an analysis of covariance.

Niche variation in the biofilm community was assessed using stable isotopes under a similar conceptual basis as Bolnick et al. (, 2007) . We used six community-level metrics described by Layman et al. (, 2007), briefly: 1) $\delta^{13}C$ range (CR), which indicates the quantity of basal resources and niche diversification at the base of the food web, 2) $\delta^{15}N$ range (NR), which shows the degree of trophic diversity, 3) total area (TA), which reflects the amount of niche space occupied by a community, 4) mean distance to centroid (CD), which shows the overall degree of trophic diversity, and is specially useful in cases with outlier species, 5) mean nearest neighbour distance (NND), which is a proxy of trophic redundancy and 6) standard deviation of the nearest neighbour distance (SDNND), which indicates the evenness of the distribution of trophic niches in a community. For a description of the calculations of each metric and a more thorough explanation see Layman (, 2007).

In order to integrate the several parameters measured we used multivariate analyses techniques using the program Primer (v. 6) and its complementary statistical package PERMANOVA+ (v. 1). A PERMDISP (Distance-based test for homogeneity of multivariate dispersions) analysis was used to measure the dispersion of $\delta^{13}C$ and $\delta^{15}N$, considered together, in all the stations for both seasons. Before the PERMDISP routine, a resemblance matrix was calculated using Euclidean distances following the recommendations of Clarke and Gorley (2006) for environmental samples. The analysis comprised 9999 permutations, using as measures distances to centroid and obtaining p-values from permutations. PERMDISP was run at different levels in the design to clarify dispersion effects following the recommendations of Anderson et al. (, 2008). First PERMDISP was run within each sampling stations individually without considering seasonality (i.e. combining the two factors, station and season). By doing this, we analysed the homogeneity of multivariate dispersion within sampling stations for both seasons. Then, PERMDISP was run between sampling stations considering each season (taking season as a higher factor). With the latter analysis, we used multivariate dispersion as a test for similarity in trophic diversity between the two seasons.

For each season, stations (samples) were ordinated according to the following variables: TOC, TON, TP, Se, Fe, Al, Mn, Cu, Zn, Cd, Pb, Ni, As, Cr and Tl concentrations using a Principal Component Analysis (PCA) routine. The data had been previously normalized to avoid skewness in the analysis due to different element concentration ranges. The obtained eigenvectors, PC1 and PC2, for each variable were plotted to see the accumulation patterns of the analyzed elements with distance from the fish farm. Then, a resemblance matrix using the normalized data was obtained using Euclidean distances following Clarke and Gorley (, 2006) recommendations for environmental samples. All statistical tests were performed with a significance level of $\alpha = 0.05$.

RESULTS

During the retrieval of the glass slides in all the station for both seasons, no macroscopic grazers were observed, indicating that the possible effect of these organisms on modifying the biofilm community was minimum.

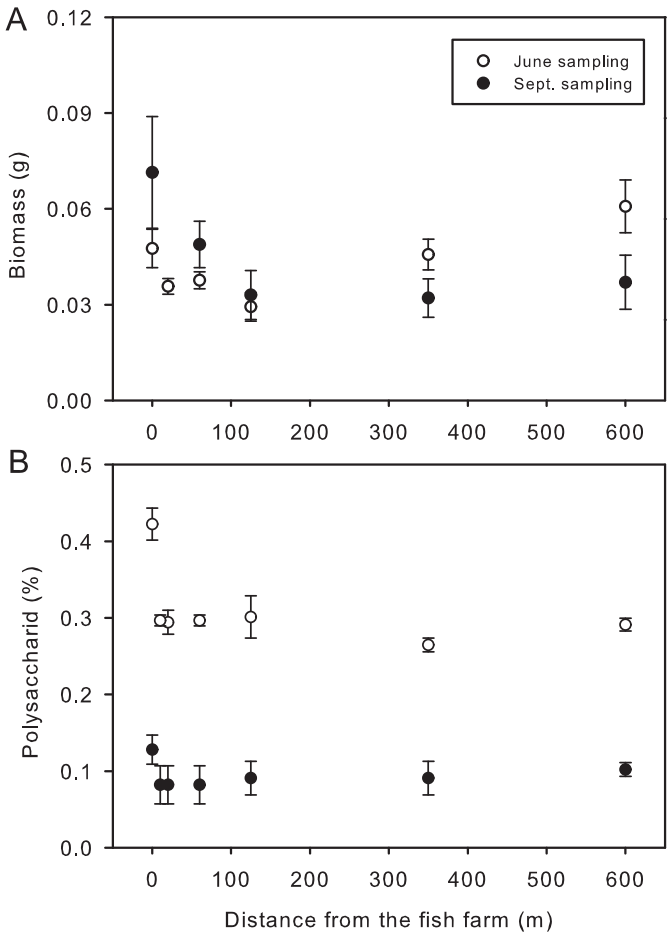

Figure 1. A) Dry weight biomass and B) polysaccharide content (mean ± SE; n=4) in the biofilm community in June (○) and September (●) along the spatial gradient from the fish farm.

Biofilm structure

Dry weight biomass of the biofilm community ranged from 0.029 ± 0.004 to 0.061 ± 0.008 g and from 0.032 ± 0.006 to 0.071 ± 0.018 g for June and September samplings, respectively (values expressed like this are always mean ± SE). Biomass was significantly higher in the station at 0 m compared with the station at 600 m from the fish farm only in September. The trend of the biofilm community biomass with

distance differed significantly between both seasons (Table 1). In the June sampling, biomass decreased with distance from the fish farm up to 120 m and then increased, while in September the biomass showed a continuous decrease with distance from the fish farm (Fig. 1 A).

	ANOVA (main test)			ANOVA (pair wise test)				Slope differences (June vs September)
Parameter	Distance	Season	Interaction	0 vs 0 m	600 vs 600 m	0 vs 600 m in June	0 vs 600 m in September	
Biomass	<0.05	n.s.	n.s.	n.s.	n.s.	n.s.	< 0.05	<0.01
Polysac.	< 0.0001	< 0.0001	<0.001	<0.001	<0.001	<0.001	n.s.	n.s.
TOC	< 0.0001	< 0.0001	< 0.0001	ns	< 0.05	<0.001	<0.001	n.s.
TON	< 0.0001	< 0.0001	< 0.0001	<0.001	<0.001	<0.001	<0.001	n.s.
PT	< 0.0001	< 0.0001	< 0.0001	<0.001	<0.001	< 0.05	<0.001	< 0.0001
$\delta^{13}C$	< 0.0001	< 0.0001	< 0.0001	<0.001	n.s.	<0.001	<0.001	<0.05
$\delta^{15}N$	< 0.0001	n.s.	< 0.0001	<0.001	n.s.	<0.001	< 0.05	<0.01

n. s. = non-significant

Table 1. Results of the two-way ANOVA and analysis of the slope (June vs September sampling) of the regressions of dry weight biomass, polysaccharide content, total organic carbon, total organic nitrogen, total phosphorous, $\delta^{13}C$ and $\delta^{15}N$ of the biofilm community along the spatial transect from the fish farm in June and September. All values are expressed as P values.

The polysaccharide content of the biofilm community was much higher in June, when it ranged from 0.26 ± 0.01 to 0.42 ± 0.02 % than in September (0.08 ± 0.02 to 0.13 ± 0.02 %). The polysaccharide content was significantly higher at 0 than at 600 m in June, while there were no significant differences between the same stations in September. In both seasons the polysaccharide content showed a tendency to decrease with distance form the fish farm. The differences were more marked in June (Fig. 1 B), although not to a statistically significant extent (Table 1).

The biofilm polysaccharide content in both seasons (June vs September) was not correlated (R^2 = 0.787 , n = 16 , p = 0.113, Fig. 1 A); nor was the polysaccharide content and biofilm biomass (polysaccharide vs biomass) for either season (June: R^2 = 0.020, n = 18 , p = 0.803; September: R^2 = 0.04 , n = 12 , p = 0.917, Fig. 1).

Biofilm trophic niche

The isotopic signatures clearly differentiated the biofilm community from the fish feed (Fig. 2 A). In fish feed, $\delta^{13}C$, was -22.2 ± 0.06 ‰, the lowest value of all the samples, and showed a significantly different accumulation in the biofilm community during both seasons (Fig. 2 B, Table 1).

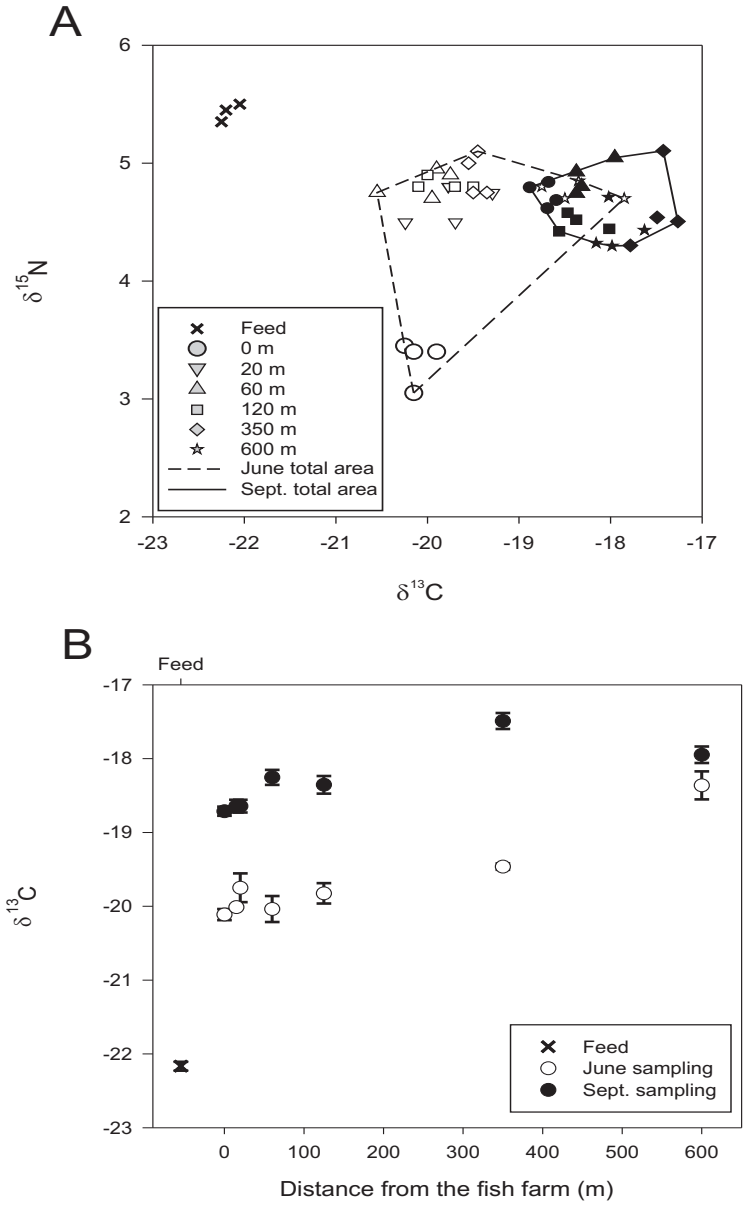

Figure 2. A) $\delta^{13}C$ and $\delta^{15}N$ in the fish feed (×) and the biofilm community at June (empty symbols) and at September (solid symbols) at 0 (○), 20 (▽), 60 (△), 120 (□), 350 (◇) and 600 m (☆) from the studied fish farm. Lines show the isotopic niche widths of the biofilm community in all the sampling stations for June (dashed line) and September (solid line) as total area. B) $\delta^{13}C$ in the fish feed (×) (n=3, mean ± SE) and the biofilm community (n=4, mean ± SE) in June (○) and September (●) along the spatial gradient from the fish farm.

$\delta^{13}C$ variation in the biofilm community ranged from -20.6 to -17.9 ‰, and from -18.9 to -17.3 ‰ for June and September, respectively (Fig. 2 A). The $\delta^{13}C$ content was significantly lower at 0 than at 600 m from the fish farm in both seasons. Similarly, there were significant differences between the stations placed 0 m from the fish farm in the two seasons, but not between the sampling stations at 600 m (Table 1).

In fish feed, $\delta^{15}N$ was 5.4 ± 0.04 ‰ which was the highest value recorded of all the samples. In the biofilm community, it varied little between sampling stations being in most cases close to 5 ‰, except in the station 0 m from the fish farm in June, when the values were markedly lower (3.3 ± 0.1 ‰). Thus, the trend in $\delta^{15}N$ accumulation with distance was significantly different between both seasons (Table 1). The variation in $\delta^{15}N$ was between 3.1 and 5.1 ‰ and between 4.3 and 5.0‰ for June and September, respectively (Fig. 2 A). The $\delta^{15}N$ content was significantly lower at 0 than at 600 m from the fish farm in both seasons. There were also significant differences between the stations placed at 0 m from the fish farm in both seasons, but no significant differences between the sampling stations located at 600 m in the two seasons (Table 1).

In the biofilm community, the $\delta^{13}C$ range (CR) and, especially, the $\delta^{15}N$ range (NR) for the biofilm community was much wider in June than in September. According to total area (TA), the trophic niche of the biofilm community was four times greater in June than in September. The mean distance to the centroid (CD) was greater in September than in June, while the mean nearest neighbour distance (NND) and the standard deviation of the nearest neighbour distance (SDNND) showed similar values: 0.179 and 0.128, and 0.186 and 0.103, for June and September, respectively (Table 2). Data for $\delta^{13}C$ and $\delta^{15}N$ indicated that the changes in the trophic niche of the biofilm community were influenced by fish farming as well as by seasonality.

PERMISP analysis of all the sampling stations regardless of the season indicated that the homogeneity of multivariate dispersion was not significantly different within stations ($p=0.794$), while the same routine between the sampling stations as regards

season indicated that multivariate dispersion was significantly higher in June than in September.

	CR	NR	TA	CD	NND	SDNND	C centroid	N centroid
Feed	0.2	0.15	0.006	0.096	0.127	0.027	-22.17	5.43
June	2.700	2.050	2.810	0.719	0.179	0.128	-19.59	4.55
September	1.621	0.807	0.885	0.460	0.186	0.103	-18.15	4.63

Table 2: Isotopic metrics for feed and the sampling stations in June and September. $\delta^{13}C$ range (CR), $\delta^{15}N$ (NR), total area (TA), mean distance to centroid (CD), mean nearest neighbor distance (NND) and standard deviation of the nearest neighbour distance (SDNND).

Biofilm elemental analysis

With the purpose of studying element accumulation in biofilm, all the elements measured were analyzed using a PCA routine. PC1 explained 51 % of the variation and grouped TOC, TON, TP, Cu, Cd, Se and Zn on the one hand and the rest of the elements on the other hand. PC2 explained 27 % of the variation and gathered Fe and Zn in one group and the rest of the elements in another. Taking into consideration both axes of the PCA, TOC, TON, Cu, TP, Se and Cd gathered together, while Pb, Tl, Ni, Cr, Al, Mn and As formed another group. Fe and Zn were considerably distant from the rest of the elements (Fig. 3).

The TOC content of the biofilm community ranged from 13.4 ± 0.2 to 23.2 ± 0.6 % and from 14.2 ± 0.2 to 24.3 ± 1.2 % for June and September, respectively (Fig. 4 A). There was a significant diminution of TOC with increasing distance from the fish farm, but there were no significant differences between sampling times. Neither were there significant differences in TOC concentrations between seasons in the biofilm community placed at 0 m, but there were significant differences for the one placed at 600 m (Table 1).

TON concentration of the biofilm community was between 2.1 ± 0.09 and 3.9 ± 0.08 % and 2.8 ± 0.05 and 5.3 ± 0.29 % for June and September sampling, respectively (Fig. 4 B). As with TOC, there were no significant differences in the trend of the TON content between both seasons. In both seasons there was a significant decrease in TON with distance from the fish farm, although, in this case, the September values were always higher compared with June (Fig. 4 B, Table 1).

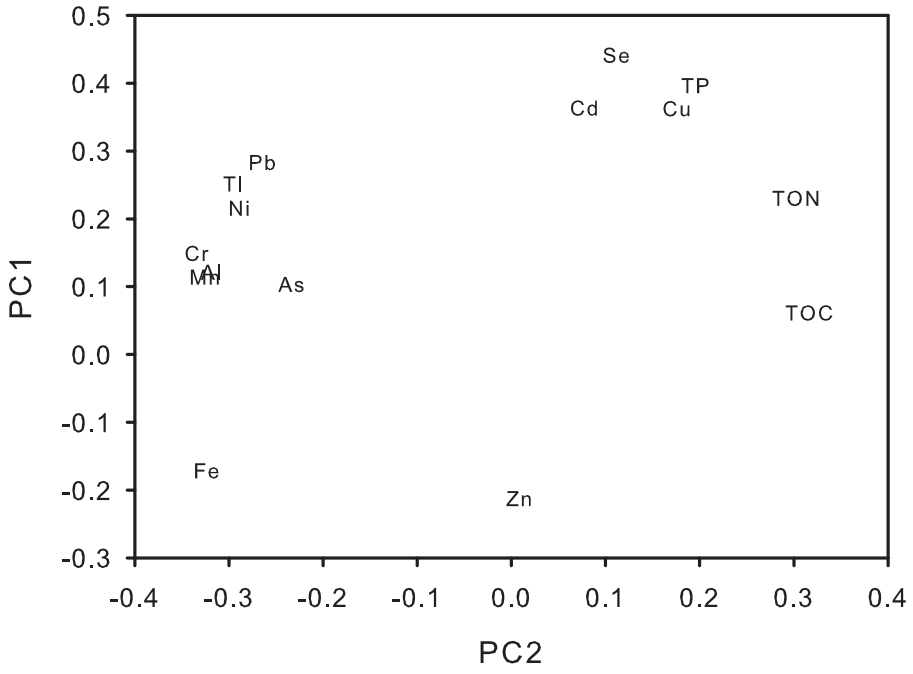

Fig. 3. Principal Component Analysis (PCA) ordination plot of PC1 and PC2 based on the concentration of the analyzed elements in the biofilm community in June and September.

The TP content of the biofilm community ranged between 0.11 ± 0.004 and 0.18 ± 0.008 % and between 0.40 ± 0.025 and 0.77 ± 0.017 % for June and September, respectively. The accumulation trend of TP was significantly different along the spatial gradient between both seasons. There was a significant decrease in the TP content between the sampling stations placed at 0 and 600m from the fish farm in both seasons, although the values were much higher in September and so the decrease between the stations furthest from each other were more marked (Fig. 4 C, Table 1).

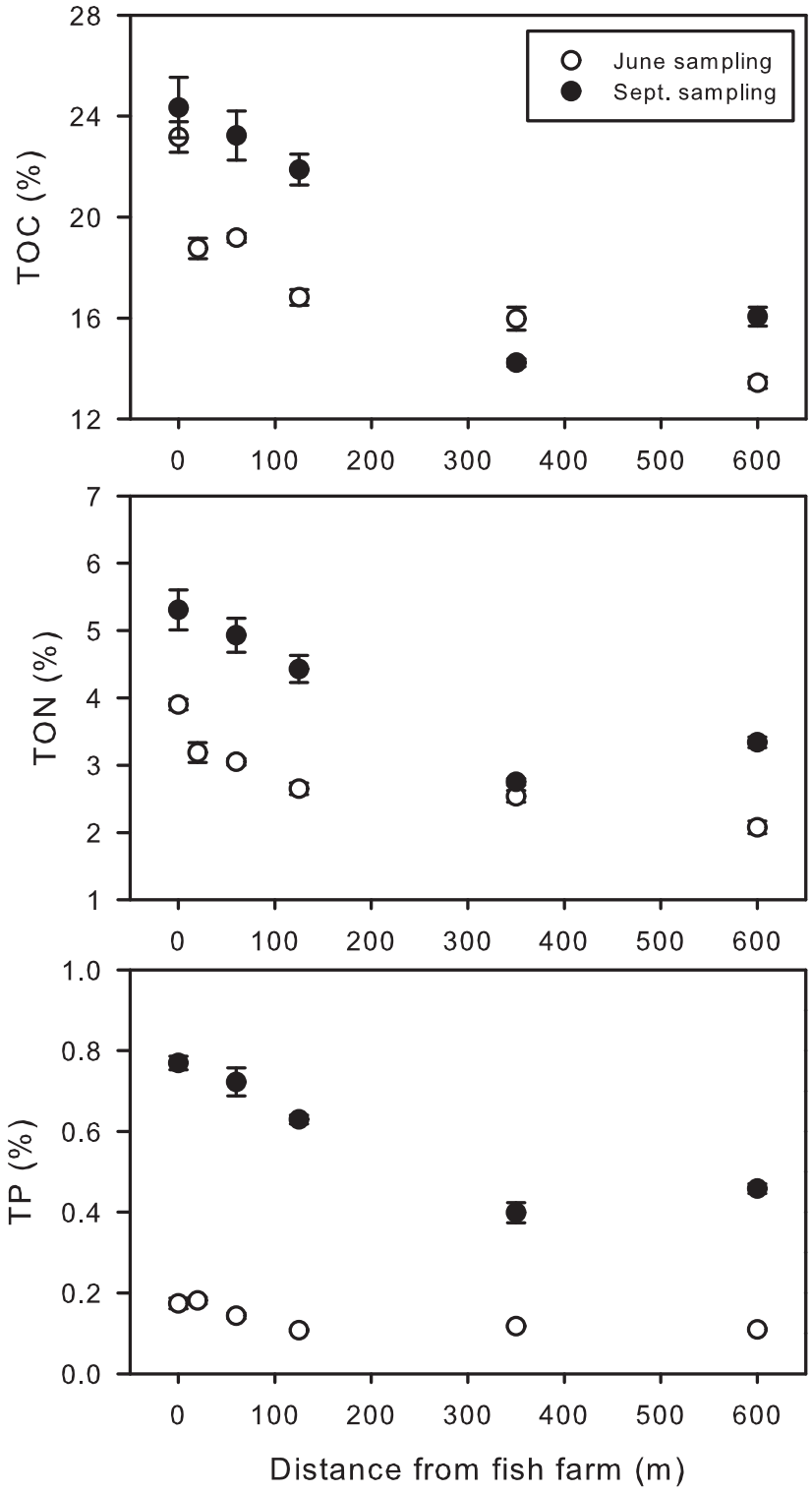

Fig. 4. A) Total organic carbon (TOC), B) total organic nitrogen (TON) and C) total phosphorous (TP) concentration (n=4, mean ± SE) in the biofilm community in June (○) and September (●) along the spatial gradient from the fish farm.

The POC, PON and TP ratios in the fish feed were similar to those found in biofilm in both seasons. Of all the elements measured in the fish feed, TOC was the most abundant while Tl was the least abundant. The most abundant metal was Fe, followed by Zn, Al, Mn, Cu and As. The rest of the elements had a concentration below 1 μg g^{-1} (Table 3).

A comparative water analysis showed that only TP, Cu, As Cr, Mn, Ni, and Tl were above the detection limits, ranging between 51.3 and 73.7, 0 and 2.5, 2.9 and 3.6, 1.0 and 1.5, 0 and 0.5, 0 and 0.9, 0.01 and 0.05, □g L^{-1}, respectively. In most of the cases, the concentrations were at least four magnitude orders lower than the concentrations in biofilm. Of these elements, only TP, Mn and Tl concentrations in the water column showed to some extent a decreasing trend with increasing distance from the fish farm.

Element	Feed concentration
C	489833 ± 4667
N	68667 ± 1014
P	9447 ± 27.1
Fe	286.9 ± 8.7
Zn	83.71 ± 0.16
Al	57.80 ± 3.63
Mn	29.74 ± 2.66
Cu	8.41 ± 0.06
As	1.63 ± 0.004
Ni	0.94 ± 0.016
Se	0.46 ± 0.006
Cr	0.33 ± 0.018
Cd	0.32 ± 0.007
Pb	0.11 ± 0.008
Tl	0.01 ± 0.0003

Table 3. Element concentration (μg g^{-1}) in the fish feed supplied to the cultured fish in the studied fish farm. The values are in dry weight (mean ± SE; n=3).

DISCUSSION

According to the biofilm structure, the biofilm biomass showed quite constant values along the environmental gradient from the fish farm in both seasons. Only the station at 0 m in September showed a higher value, being significantly greater than the station located at 600 m in the same time period (Fig. 1 A). This suggests that biomass only responds significantly at high organic matter loads, which agrees with previous works that shows than eutrophication enhances biofilm biomass (Dodds et al., 2000 and references therein).

The polysaccharide content, as an indirect measure of the extracellular polymeric substances, seemed to be quite constant with distance form the fish farm, indicating that the polysaccharide content was little influenced by the fish farm load (Fig. 1 B). However, the polysaccharide content of biofilm showed a seasonal behaviour and greater values were observed in June than in September. This could be due to the high variability in the composition, structure and amount of the extracellular polymeric substances in different microorganisms that produce it (Fig 1 B Tago and Aida, 1977; Decho, 1994). The seasonally different species composition of the biofilm community may have led to different polysaccharide contents (Riedel et al., 2007; Yucel-Gier et al., 2008).

The analysis $\delta^{13}C$ and $\delta^{15}N$ clearly separated fish feed from the biofilm community samples (Fig. 2 A). The biofilm isotopic C and N signature was within as similar range to that recorded in other studies (Kuwae et al., 2008; Marin-Guirao et al., 2008). Fish feed showed the lowest $\delta^{13}C$ values, indicating a more terrestial source, which could be due to the terrestrial components of the fish feed. In both seasons, the $\delta^{13}C$ signature in the biofilm community was more influenced by fish feed in the stations at 0 m than at 600 m, showing the $\delta^{13}C$ content to increase with distance from the fish farm (Fig 2 B).

Biofilm is a complex community composed of autotrophic and heterotrophic organisms with different trophic levels. Thus, the $\delta^{15}N$ content of biofilm is an average of the trophic levels of the predominant organisms in biomass. In the present study, the $\delta^{15}N$ content was similar for all the samples except that at 0 m in June, which was

markedly lower (Fig. 2 A). The lower $\delta^{15}N$ content in the station at 0 m in June could be due to the presence of more autotrophic organisms than in the rest of the stations.

The biofilm community mainly assimilates dissolved elements (Lock and Ford, 1985) and the similar values between fish feed and biofilm as regards $\delta^{15}N$ confirmed that the biofilm community does not directly assimilate fish feed in its particulate and untransformed form.

As regards isotopic metrics, the total area (TA) and the mean distance to the centroid (CD) were notably greater in June than in September, indicating that the trophic diversity of the biofilm community along the spatial transect was higher in June than in September. PERMISP analysis of all the sampling stations, regardless the season, indicated that the homogeneity of multivariate dispersion was not significantly different within stations, but was significantly different when the season was considered as a factor. This fact indicated that the variability of the trophic diversity was consistent within stations but was significantly different between stations of the different seasons. So the differences in the total area (TA) and the mean distance to centroid (CD), in both seasons, were due to a significantly higher trophic diversity between stations in June than in September, and not to a high dispersion of replicates within each station.

The similar values of the mean nearest neighbour distance (NND) and the standard deviation of the nearest neighbour distance (SDNND) of the biofilm community for both seasons showed that trophic redundancy was comparable, as was the evenness of the distribution of trophic niches in both times of the year (Table 2). According to the results of this study, both season and the fish farm waste load influenced the trophic niche of the biofilm community.

As regards element accumulation by biofilm, the PCA analysis grouped stations following the environmental gradient in both seasons, indicating that the accumulation pattern in biofilm (either positive or negative) was consistent along the fish farm influence for each season. Cu, Zn and Cd seem to be the main metals released

to the environment due to fish farm activities (Dean et al., 2007; Basaran et al., 2010). In the present work, according to PCA, Cu, Cd, Se, and, to some extent Zn, seem to have a similar accumulation dynamics as TOC, TON and TP along the distance gradient (Fig. 3). Cu, Zn and Se are micronutrients which can be toxic at high levels, while Cd is a non-essential element that competes for calcium enzymatic locations (Friberg et al., 1979).

TOC, TON and TP content in the biofilm community showed a trend of exponential decay with distance, although the magnitudes were greater in September than in June, in agreement with the high production in September compared to June due to higher sea water temperatures at this time of the year. This difference between both periods was especially marked for TP (Fig. 4). Fish farm inputs of dissolved organic matter and nutrients were clearly reflected in the TOC, TON and TP contents in the biofilm community. According to the results, the effect of dissolved aquaculture wastes could be noted from 0 up to a point of 120-350 m from the fish farm. The element accumulation pattern seemed to follow the same trend in both seasons, and was consistent with the organic matter load, the accumulation in the biofilm community being higher in September, when the fish farm waste load was higher.

Studying natural biofilms can be problematic, especially when the investigation requires measuring biofilms at a variety of sites. Artificial substrates made of the same material allow us to increase the reproducibility between sites and minimize confounding influences. Similarly, as biofilms are freshly grown, the results are not confounded by different ages of the biofilm community at the different sites, nor difference in antecedent conditions (Baldwin et al., 2006).

The studied biofilm community showed a high capacity to concentrate elements from the water column released from fish farm activity. This may be attributed to the extracellular polymeric substances and the components of biofilm, which, due to their physical nature, have great adsorptive capabilities (Decho, 1990). According to the results of the present study, element accumulation by biofilm was not correlated with the polysaccharide content of the community, suggesting that the capacity to

concentrate these elements was independent of the amount of the extracellular polymeric substances.

This work also shows that the biofilm community can account for the capacity "to memorize" a disturbance effect, which the pelagic community lacks (Brummer et al., 2003). Furthermore, even though both, season and the fish farm waste load, influenced the biomass, polysaccharide content and the trophic niche of the biofilm community, the element accumulation pattern seemed to follow the same trend in both seasons being the accumulation consistent with the fish farm waste load. Hence, the biofilm community can be considered a reliable tool for assessing the reach and extent of dissolved aquaculture wastes. Due to the ubiquity of biofilm, its adsorptive capacity and its wide range of consumers, the role of biofilm as a sink of aquaculture dissolved wastes may have important implications for the transfer of these wastes to higher trophic levels in coastal systems.

CONCLUSIONS

This work demonstrates that the biofilm community is sensitive to fish farm influence, and undergoes structural and trophic changes in response. Biofilm structure and trophic diversity was influenced by seasonality as well as by the fish farm waste load. Fish farming enhanced the accumulation of TOC, TON, TP, Se and metals by the biofilm community. The accumulation pattern of these elements was similar regardless of the structure and trophic niche of the community. This suggests that the biofilm community can be considered a reliable tool for assessing dissolved aquaculture wastes and may have important implications for the transfer of aquaculture wastes to higher trophic levels in coastal systems.

Acknowledgements

We would like to express our gratitude to Culmarex S.A. company, especially M. C. Marín, M. Azzaydi, M.D. López-Belluga and all the fish farm staff who kindly allowed us to perform the study. We should also like to thank C. Gutierrez-Cánovas for his advice with the statistics. This work was funded by the project AGL2004-08350-C02-01/ACU from the Ministerio de Ciencia y Tecnología, a predoctoral grant to C. S. from the Ministerio de Educación y Ciencia and another predoctoral grant to F. N. from the ALBAN programme, The European Union Programme of High Level Scolarships for Latin America (Grant No. E07D401236EC).

REFERENCES

Abreu, P.C., Ballester, E.L.C., Odebrecht, C., Wasielesky, W., Cavalli, R.O., Graneli, W., Anesio, A.M., 2007. Importance of biofilm as food source for shrimp (Farfantepenaeus paulensis) evaluated by stable isotopes (delta C-13 and delta N-15). Journal of Experimental Marine Biology and Ecology 347, 88-96.

Admiraal, W., Blanck, H., Buckert-De Jong, M., Guasch, H., Ivorra, N., Lehmann, V., Nystrom, B.A.H., Paulsson, M., Sabater, S., 1999. Short-term toxicity of zinc to microbenthic algae and bacteria in a metal polluted stream. Water Research 33, 1989-1996.

Aldridge, K.T., Brookes, J.D., Ganf, G.G., 2010. Changes in abiotic and biotic phosphorus uptake across a gradient of stream condition. River Research and Applications.

Allison, D.G., Gilbert, P., 1992. Bacterial Biofilms. Science Progress 76, 305-321.

Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. PERMANOVA + for PRIMER: Guide to Software and Statistical Methods. PRIMER-E, Plymouth, UK.

Azam, F., 1998. Microbial control of oceanic carbon flux: The plot thickens. Science 280, 694-696.

Azam, F., Smith, D.C., Steward, G.F., Hagstrom, A., 1994. Bacteria - Organic-Matter Coupling and Its Significance for Oceanic Carbon Cycling. Microbial Ecology 28, 167-179.

Baird, B.H., Thistle, D., 1986. Uptake of Bacterial Extracellular Polymer by A Deposit-Feeding Holothurian (Isostichopus-Badionotus). Marine Biology 92, 183-187.

Baldwin, D.S., Mitchell, A.M., Rees, G.N., Watson, G.O., Williams, J.L., 2006. Nitrogen processing by biofilms along a lowland river continuum. River Research and Applications 22, 319-326.

Barranguet, C., Plans, M., van der Grinten, E., Sinke, J.J., Admiraal, W., 2002. Development of photosynthetic biofilms affected by dissolved and sorbed copper in a eutrophic river. Environmental Toxicology and Chemistry 21, 1955-1965.

Basaran, A.K., Aksu, M., Egemen, O., 2010. Impacts of the fish farms on the water column nutrient concentrations and accumulation of heavy metals in the sediments in the eastern Aegean Sea (Turkey). Environmental Monitoring and Assessment 162, 439-451.

Battin, T.J., Kaplan, L.A., Newbold, J.D., Hansen, C.M.E., 2003. Contributions of microbial biofilms to ecosystem processes in stream mesocosms. Nature 426, 439-442.

Bolnick, D.I., Svanback, R., Araujo, M.S., Persson, L., 2007. Comparative support for the niche variation hypothesis that more generalized populations also are more heterogeneous. Proceedings of the National Academy of Sciences of the United States of America 104, 10075-10079.

Brummer, I.H.M., Felske, A., Wagner-Dobler, I., 2003. Diversity and seasonal variability of beta-proteobacteria in biofilms of polluted rivers: Analysis by temperature gradient gel electrophoresis and cloning. Applied and Environmental Microbiology 69, 4463-4473.

Characklis, W.G., Marshall, K.C., 1990. Biofilms: A basis for and interdisciplinary approach. In: W. G. Characklis and K. C. Marshall (Eds.). Wiley-Interscience, New York, pp. 3-16.

Cifuentes, L.A., Sharp, J.H., Fogel, M.L., 1988. Stable Carbon and Nitrogen Isotope Biogeochemistry in the Delaware Estuary. Limnology and Oceanography 33, 1102-1115.

Clarke, K.R., Gorley, R.N., 2006. Primer v6: User Manual/Tutorial. PRIMER-E Ltd, Plymouth, UK.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., Lappinscott, H.M., 1995. Microbial Biofilms. Annual Review of Microbiology 49, 711-745.

Danovaro, R., 2003. Pollution threats in the mediterranean sea: an overview. Chemistry and Ecology 19, 15-32.

Darimont, C.T., Paquet, P.C., Reimchen, T.E., 2009. Landscape heterogeneity and marine subsidy generate extensive intrapopulation niche diversity in a large terrestrial vertebrate. Journal of Animal Ecology 78, 126-133.

Das, B.K., Roy, A., Koschorreck, M., Mandal, S.M., Wendt-Potthoff, K., Bhattacharya, J., 2009. Occurrence and role of algae and fungi in acid mine drainage environment with special reference to metals and sulfate immobilization. Water Research 43, 883-894.

Dean, R.J., Shimmield, T.M., Black, K.D., 2007. Copper, zinc and cadmium in marine cage fish farm sediments: An extensive survey. Environmental Pollution 145, 84-95.

Decho, A.W., 1990. Microbial Exopolymer Secretions in Ocean Environments - Their Role(S) in Food Webs and Marine Processes. Oceanography and Marine Biology 28, 73-153.

Decho, A.W., 1994. Molecular-scale events influencing the macroscale cohesiveness of exopolymers. In: W. E. Krumbein, D. M. Paterson and L. J. Stal (Eds.). Biostabilization of sediments. Verlag, Oldenburg, pp. 135-148.

Decho, A.W., Moriarty, D.J.W., 1990. Bacterial Exopolymer Utilization by A Harpacticoid Copepod - A Methodology and Results. Limnology and Oceanography 35, 1039-1049.

Deegan, L.A., Garritt, R.H., 1997. Evidence for spatial variability in estuarine food webs. Marine Ecology-Progress Series 147, 31-47.

Dodds, W.K., Evans-White, M.A., Gerlanc, N.M., Gray, L., Gudder, D.A., Kemp, M.J., Lopez, A.L., Stagliano, D., Strauss, E.A., Tank, J.L., Whiles, M.R., Wollheim, W.M., 2000. Quantification of the nitrogen cycle in a prairie stream. Ecosystems 3, 574-589.

Friberg, L., Norberg, G.F., Vouk, V.B., 1979. Handbook of metal toxicology. Elsevier/North Holland, Amsterdam.

Hynes, H.B.N., 1970. The ecology. Liverpool University Press, Liverpool.

Ivorra, N., Hettelaar, J., Tubbing, G.M.J., Kraak, M.H.S., Sabater, S., Admiraal, W., 1999. Translocation of microbenthic algal assemblages used for in situ analysis of metal pollution in rivers. Archives of Environmental Contamination and Toxicology 37, 19-28.

Kalantzi, I., Karakassis, I., 2006. Benthic impacts of fish farming: Meta-analysis of community and geochemical data. Marine Pollution Bulletin 52, 484-493.

Karakassis, I., Tsapakis, M., Hatziyanni, E., Papadopoulou, K.N., Plaiti, W., 2000. Impact of cage farming of fish on the seabed in three Mediterranean coastal areas. Ices Journal of Marine Science 57, 1462-1471.

Khatoon, H., Yusoff, F., Banerjee, S., Shariff, M., Bujang, J.S., 2007. Formation of periphyton biofilm and subsequent biofouling on different substrates in nutrient enriched brackishwater shrimp ponds. Aquaculture 273, 470-477.

Kuwae, T., Beninger, P.G., Decottignies, P., Mathot, K.J., Lund, D.R., Elner, R.W., 2008. Biofilm grazing in a higher vertebrate: The Western Sandpiper, Calidris mauri. Ecology 89, 599-606.

Layman, C.A., Arrington, D.A., Montana, C.G., Post, D.M., 2007. Can stable isotope ratios provide for community-wide measures of trophic structure? Ecology 88, 42-48.

Lock, M.A., Ford, T.E., 1985. Microcalorimetric Approach to Determine Relationships Between Energy Supply and Metabolism in River Epilithon. Applied and Environmental Microbiology 49, 408-412.

Marin-Guirao, L., Llotet, J., Marin, A., 2008. Carbon and nitrogen stable isotopes and metal concentration in food webs from a mining-impacted coastal lagoon. Science of the Total Environment 393, 118-130.

Morin, S., Duong, T.T., Dabrin, A., Coynel, A., Herlory, O., Baudrimont, M., Delmas, F., Durrieu, G., Schafer, J., Winterton, P., Blanc, G., Coste, M., 2008. Long-term survey of heavy-metal pollution, biofilm contamination and diatom community structure in the Riou Mort watershed, South-West France. Environmental Pollution 151, 532-542.

Morrisey, D.J., Gibbs, M.M., Pickmere, S.E., Cole, R.G., 2000. Predicting impacts and recovery of marine-farm sites in Stewart Island, New Zealand, from the Findlay-Watling model. Aquaculture 185, 257-271.

Navarro, N., Leakey, R.J.G., Black, K.D., 2008. Effect of salmon cage aquaculture on the pelagic environment of temperate coastal waters: seasonal changes in nutrients and microbial community. Marine Ecology-Progress Series 361, 47-58.

Neofitou, N., Klaoudatos, S., 2008. Effect of fish farming on the water column nutrient concentration in a semi-enclosed gulf of the Eastern Mediterranean. Aquaculture Research 39, 482-490.

Pacepavicius, G., Lau, Y.L., Liu, D., Okamura, H., Aoyama, I., 1997. A rapid biochemical method for estimating biofilm mass. Environmental Toxicology and Water Quality 12, 97-100.

Pitta, P., Apostolaki, E.T., Tsagaraki, T., Tsapakis, M., Karakassis, I., 2006. Fish farming effects on chemical and microbial variables of the water column: A spatio-temporal study along the Mediterranean Sea. Hydrobiologia 563, 99-108.

Pitta, P., Karakassis, I., Tsapakis, M., Zivanovic, S., 1998. Natural vs. mariculture induced variability in nutrients and plankton in the eastern Mediterranean. Hydrobiologia 391, 181-194.

Pitta, P., Tsapakis, M., Apostolaki, E.T., Tsagaraki, T., Holmer, M., Karakassis, I., 2009. 'Ghost nutrients' from fish farms are transferred up the food web by phytoplankton grazers. Marine Ecology-Progress Series 374, 1-6.

Pusceddu, A., Fraschetti, S., Mirto, S., Holmer, M., Danovaro, R., 2007. Effects of intensive mariculture on sediment biochemistry. Ecological Applications 17, 1366-1378.

Quigley, M.S., Santschi, P.H., Hung, C.C., Guo, L.D., Honeyman, B.D., 2002. Importance of acid polysaccharides for Th-234 complexation to marine organic matter. Limnology and Oceanography 47, 367-377.

Rao, T.S., Rani, P.G., Venugopalan, V.P., Nair, K.V.K., 1997. Biofilm formation in a freshwater environment under photic and aphotic conditions. Biofouling 11, 265-282.

Riedel, A., Michel, C., Gosselin, M., Leblanc, B., 2007. Enrichment of nutrients, exopolymeric substances and microorganisms in newly formed sea ice on the Mackenzie shelf. Marine Ecology-Progress Series 342, 55-67.

Smith, D.J., Underwood, G.J.C., 1998. Exopolymer production by intertidal epipelic diatoms. Limnology and Oceanography 43, 1578-1591.

Stal, L.J., 2003. Microphytobenthos, their extracellular polymeric substances, and the morphogenesis of intertidal sediments. Geomicrobiology Journal 20, 463-478.

Tago, Y., Aida, K., 1977. Exocellular Mucopolysaccharide Closely Related to Bacterial Floc Formation. Applied and Environmental Microbiology 34, 308-314.

van Dam, A.A., Beveridge, M.C.M., Azim, M.E., Verdegem, M.C.J., 2002. The potential of fish production based on periphyton. Reviews in Fish Biology and Fisheries 12, 1-31.

Vis, C., Hudon, C., Cattaneo, A., Pinel-Alloul, B., 1998. Periphyton as an indicator of water quality in the St Lawrence River (Quebec, Canada). Environmental Pollution 101, 13-24.

Webster, N.S., Negri, A.P., 2006. Site-specific variation in Antarctic marine biofilms established on artificial surfaces. Environmental Microbiology 8, 1177-1190.

Yucel-Gier, G., Uslu, O., Bizsel, N., 2008. Effects of marine fish farming on nutrient composition and plankton communities in the Eastern Aegean Sea (Turkey). Aquaculture Research 39, 181-194.

Zar, J., 1984. Biostatistical Analysis. Prentice-Hall, New Jersey.

3. TROPHIC NICHE MODIFICATION OF WILD FISH COMMUNITY ASSOCIATED TO AN OPEN WATER FISH FARM

Trophic niche modification of wild fish community associated to an open water fish farm

Francisco Navarrete-Mier, Department Ecology and Hydrology, University of Murcia. Murcia, 30100, Spain.
Ander Montoya, Department of Animal Physiology, University of Murcia. Murcia, 30100, Spain.
Carlos Sanz-Lázaro, Department Ecology and Hydrology, University of Murcia. Murcia, 30100, Spain.
Arnaldo Marín, Department Ecology and Hydrology, University of Murcia. Murcia, 30100, Spain.

ABSTRACT:

1. Marine fish farms in open waters act like FADs (fish aggregating devices) maintaining high densities of wild fish permanently associated and to the net cages.
2. We analyzed the abundance of fish and the concentration of stable isotopes of fish permanently associated to fish farms in the Mediterranean area at species and community level to assess the changes on their trophic niche due to the presence of artificial feed in fish cultures.
3. Fish abundance around the farms was drastically higher compared to the areas not influenced by marine aquaculture. The concentration of stable isotopes in fish associated to farms was shown to be related to the leak of artificial feed from the farm. Further, at community level there is a clear compression of the trophic niche in those fish assemblages, being significantly different to the niches in natural communities, which are remarkably wider.
4. Considering the high and growing number of fish farms in the Mediterranean, a compression of the trophic niche in large communities of "wild" fish could imply a serious impact in the environment, particularly in the stocks of natural fish populations.

KEY WORDS:

Aquaculture, Aquatic Food Webs, Stable isotopes, Fish aggregating devices, Wild fish.

INTRODUCTION

Great amount of fish may be associated or aggregate around natural or artificial floating structures in open waters, which are known as FADs (Fish Aggregating Devices) (Castro, Santiago & Santana-Ortega 2001; Hunter & Mitchell 1968). Fish farms in open sea performs like FADs (Boyra *et al.* 2004; Dempster *et al.* 2002; Sudirman *et al.* 2009), usually aggregate great quantity of fish immediately next to net cages, and this concentration may decline 100s of meters away (Dempster *et al.* 2010). This association is ecologically important since wild fish associated, in some cases, could overcome the biomass of the cultured fish (Sudirman *et al.* 2009; Uglem *et al.* 2009). The reason of this aggregation have not been clearly defined yet, but even when the output of organic matter leak from the fish farms to the system, like fish feed, cannot sustain the great amount of associated fish, their highly attractive effect is indisputable (Bjordal & Skar 1992; Freon & Dagorn 2000). Wild fish associated to farms consume organic wastes from the fish farm in the water column and in benthos (Fernandez-Jover *et al.* 2007; Sanz-Lázaro 2010; Vita *et al.* 2004), however, the effect of wastes derived from fish farming on the trophic relationships of associated wild fish communities has not been yet studied.

The differentiation between cultured fish with wild fish populations not influenced by aquaculture has been widely studied with morphological analyses (Kurtovic, Teskeredzic & Teskeredzic 2008), sensorial aspects (Grigorakis, Taylor & Alexis 2003), pollutants (Serrano, Barreda & Blanes 2008), fatty acids (Bell *et al.* 2007; Busetto *et al.* 2008), stable isotopes (Dempson & Power 2004; Moreno-Rojas *et al.* 2007), and genetic (Alarcon *et al.* 2004). There are only some studies which analyze the abundance and diversity of associated fish to a farm (Dempster *et al.* 2009; Machias *et al.* 2004; Valle *et al.* 2007). It has not been yet defined if fish associated to fish farms belonging to the same cultured species are wild fish attracted by the cages, but is highly probably that they are individuals escaped from cultures (Dempster *et al.* 2007) and usually they reach higher sizes that the cultured ones. Also, wild fish populations of species objective of culture have been poorly studied, and in some species such as European sea bass (*Dicentrarchus labrax*) or gilthead

sea bream (*Sparus aurata*) is believed to be considerably lower than the standing stock in sea-cages (ICES 2006). With the increase of mariculture, the risk of genetic contamination generated by escaped animals in the wild stocks is high, this genetic interaction could bring declines in endemic - evolutionary significant units, and considering that the gene flow is pervasive and persistent it could cause significant decreases in some wild/feral stocks (ICES 2006).

In addition to the cultured species, there are many species of wild fish associated to the farms. Even most fish species have a great mobility, some species are year-round residents of the offshore net cages although their abundance may vary with the season, this variation is not significant (Boyra *et al.* 2004). While, other associated species present at the farm sporadically and in relative low abundance are migratory and may vary his presence depending the season.

Carbon and nitrogen stable isotope analyses have emerged as reliable tools for elucidating trophic structures and inferring pathways of energy flow in food webs (Cifuentes, Sharp & Fogel 1988; Fry *et al.* 2008) especially in a variety of aquatic ecosystems (Hobson & Welch 1992; Marin-Guirao, Llotet & Marin 2008; Thornton & Mcmanus 1994). The growth of fish tissues is relatively slow (Maruyama *et al.* 2001) therefore the isotopic composition of tissues reflects a time-integrating trophic interaction (Deudero *et al.* 2004; Lefebvre, Harma & Blin 2009). White muscle of fishes is the less $\delta^{13}C$ and $\delta^{15}N$ variable tissue, for that reason is probably the best tissue for use in ecological works (Deudero et al. 2004; Lefebvre, Harma & Blin 2009; Pinnegar & Polunin 1999). The use of stable isotopes on trophic webs to assess feeding sources of a fish species would allow an analysis of their ecological niche within the system. Studies show trophic differences between cultured and wild fish (Dempson & Power 2004; Serrano, Barreda & Blanes 2008) and validate this procedure to know with certainty the origin of the fish (Moreno-Rojas *et al.* 2007) but there are no studies that analyze the wild fish community associated to a marine fish farm. The fish of littoral areas tend to show widest ranges of stable isotopes accumulation (Pinnegar & Polunin 2000) mainly in littoral systems where fish has access to a variety of primary producers with different values of $\delta^{13}C$

(Bricout 1982). The niche of wild fish may be affected by the increase of an external source of food from fish farm and modify the normal trophic relations in fish communities.

According to FAO (2006) the marine aquaculture is an industry in constant growth since 1990 and is expected to rise at least 5% annually in the next two decades, therefore the study of the impact of this activity in wild fish population is crucial to preserve the resources. In that sense this work constitutes an initial approach to assess the ecological value of these affected fish on their contribution to the environment.

The objective of this study was to determinate the trophic niche changes of wild fish associated to a fish farm, at species and community level, due to the influence of an anthropogenic activity such as marine fish farming.

MATERIALS AND METHODS

Study area. The study was conducted during the winter 2008 in the surroundings of a marine fish farm located in Águilas, SE Spain, (western Mediterranean; 37°24'56.2''N, 1°32'4.0''W) with a total annual production of 1000 tonnes. The farm produces gilthead sea bream (*S. aurata*) and European sea bass (*D. labrax*) in a 24 net cage system, each cage measuring 25 m diameter and 19 m depth. We defined four sites for analyses: (**I**) Inside the cages, (**O**) Outside the cages (ca, 100m around the net-cages), (**F**) Fraile Island (average 1200 m to the west of net-cages), and (**C**) Cape Cope (4500m to the east of the net-cages (Fig. 1).

Fish Counting. Estimates of wild fishes abundances in all sites with exception of site **I** was performed by visual techniques, since these are a non-destructive and fast methods with a high replication degree (Valle *et al.* 2007). Two divers perform triplicate 5 minutes transects within 5 meters on either side of transect recording the abundance of fish species according to Hamerlin-Vivien et al. (1985) and using

the groups technique proposed by Dempster et al. (2002) for minimize errors, in order to avoid the possibility of underestimating or overestimating the counts.

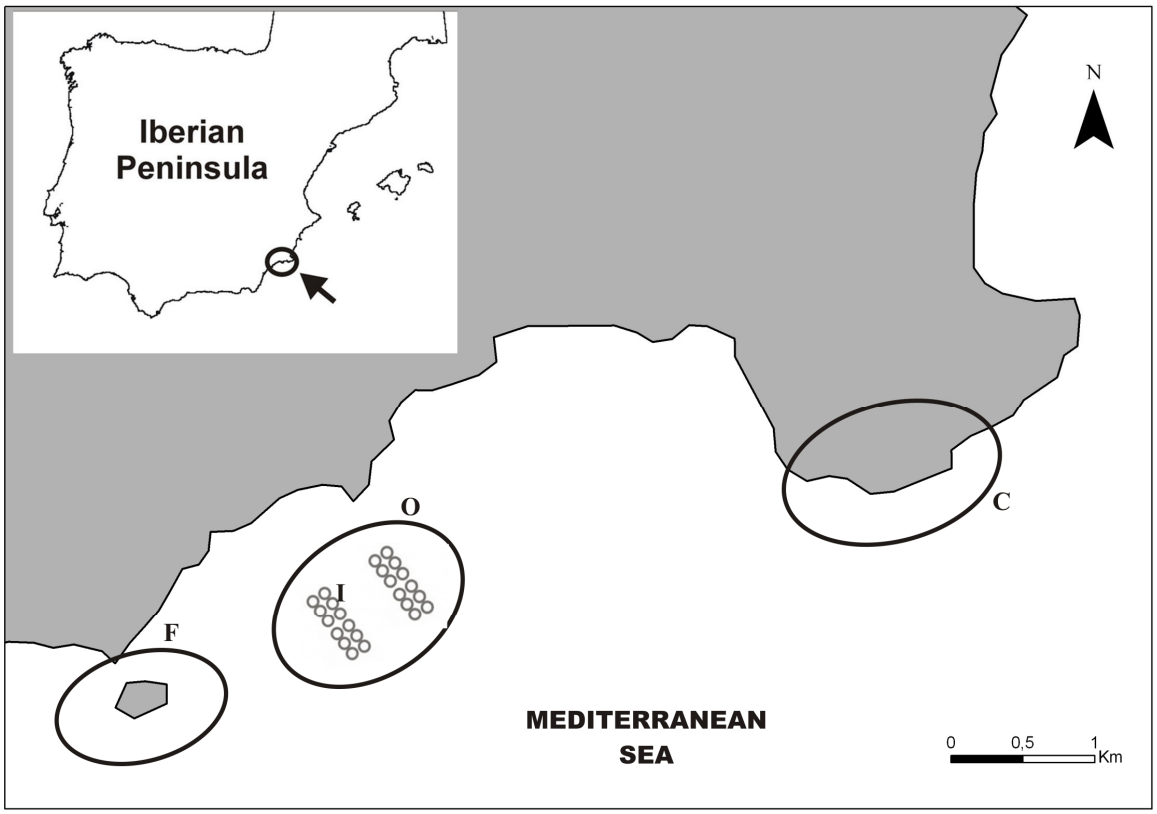

Figure 1. Localization of the studied fish farm in Murcia coast (Spain) and the sampling sites (I = inside cages, O = outside cages farm, F = Fraile Island, C = Cape Cope)

Sample collection. After the visual counts fish were collected by spear-fishing, this technique was chosen among others since it produces minimum environmental impact. We collected and analyzed ten fish species selected in the basis of their permanent residence in the area, commercial and ecological importance and the logistic feasibility to collect them. At least 3 fish were collected by species by site. For all sampled fish, the biometrical data was registered, then fish were dissected and a sample of dorsal white muscle was taken (Periago *et al.* 2005) which was stored at -20°C until analyses for stable isotopes. The absence of some species in the sites was debt to ecological differences between sampling sites (Fig.2). In addition, samples of different fish feed was obtained from the fish farm; to ensure there were no confounding effects associated with different strains of formulated feed,

three replicates of each type of fish feed were analysed to asses feed sample homogeneity. This study complies with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for using of animals in research.

Stable isotope analysis. The study was perform using white muscle samples because is the fish tissue which presents minimum variations in the concentrations of $\delta^{13}C$ and $\delta^{15}N$ compared to other tissues (Pinnegar & Polunin 1999), then it is considerate the best fish tissue for ecological stable isotope studies (Deudero *et al.* 2004; Moreno-Rojas *et al.* 2007).

All tissue and feed samples were dried by lyophilization (Carabel *et al.* 2006). Samples were pulverized to a fine powder, homogenized (Grey 2001; Jennings *et al.* 1997) and subsequently analyzed by duplicate with an elemental analyzer Flash EA1112 (ThermoFinnigan) connected to a Delta^{plus} mass spectrometer of isotopic relationships (ThermoFinnigan).

All the isotopic data are reported in the conventional δ notation as follows:

$$\delta^{13}C \text{ or } \delta^{15}N = (Rsample / Rstandar - 1)\, 1000\ (‰)$$

Where R represents the 13C/12C or 15N/14N ratio for δ^{13} C and δ^{15} N, respectively. All $\delta^{13}C$ values were reported as the deviation relative to the Vienna Pee Dee Belemnite Limestone Standart (v-PDB). The $\delta^{15}N$ standards were calibrated and results are reported relative to atmospheric nitrogen.

Stadistical analyses. To compare fish assemblages between the sites we used the fish abundance data and performed multivariate techniques using PRIMER (v. 6) (Clarke & Warwick 1994). SIMPER was used to determine the relative importance of each species of fish contributing to the dissimilarity among samples. Using the Bray-Curtis similarity coefficient we calculated the similarity matrices. Then we

performed a non-metric multidimensional scaling (MDS) and for the graphic results were presented as two-dimensional plot. Cluster classification analysis was performed to group samples in the MDS. The abundance estimation of both divers was represented separately as different replicates.

For stable isotopes analyses a one way analysis of variance (ANOVA) was performed to identify significant differences between each fish species and site studied. Normality data was checked with the Kolmogorov-Smirnov test and homogeneity of variances with the Levene test. When significant differences were found between sites, a Tukey HSD *post hoc* test, or when a species was only found at two sites, *t*-test was performed. All statistical tests were performed with a significance level of $\alpha=0.05$.

Bi-dimensional plots were constructed to represent isotope analysis data. As Lloret & Marín (2009) describe community-wide metrics calculated for each site may be used to identify possible differences in trophic diversity and/or trophic redundancy between the sites. To quantitatively asses trophic diversity of the fish community of the areas of the study, we follows the recommendations of Layman et al. (2007a) who propose six community level metrics: $\delta^{15}N$ range (NR), which shows degree of trophic diversity and vertical food web structure; $\delta^{13}C$ range (CR), which show the quantity of basal resources and niche diversification at the base of the food web; Total area (TA), defined by the minimum convex polygon bounding the most divergent individuals and represent the amount of niche space occupied by a community; Mean distance to centroid (CD) which describe the overall degree of trophic diversity; Mean nearest neighbour distance (NND), which is a proxy of trophic redundancy; Standart deviation of the nearest neighbour distance (SDNND), which indicates the evenness of the distribution of trophic niches in a community. Also we record the C centroid and N centroid, that allow us to see the spatial position of the average of the community stable isotope signal.

RESULTS

Fish communities structure

The fish counts showed clear abundance differences between the site O (1819± 278.6) and the other sites such as F (1313 ± 98.2) and C (758 ± 62.2) (Fig 2). The main contribution to fish abundance in the sites F and C was due to *Chromis chromis*, with a standard length less than 10 cm. Although in site O the fish size for most of the species was remarkably higher than in the other sites.

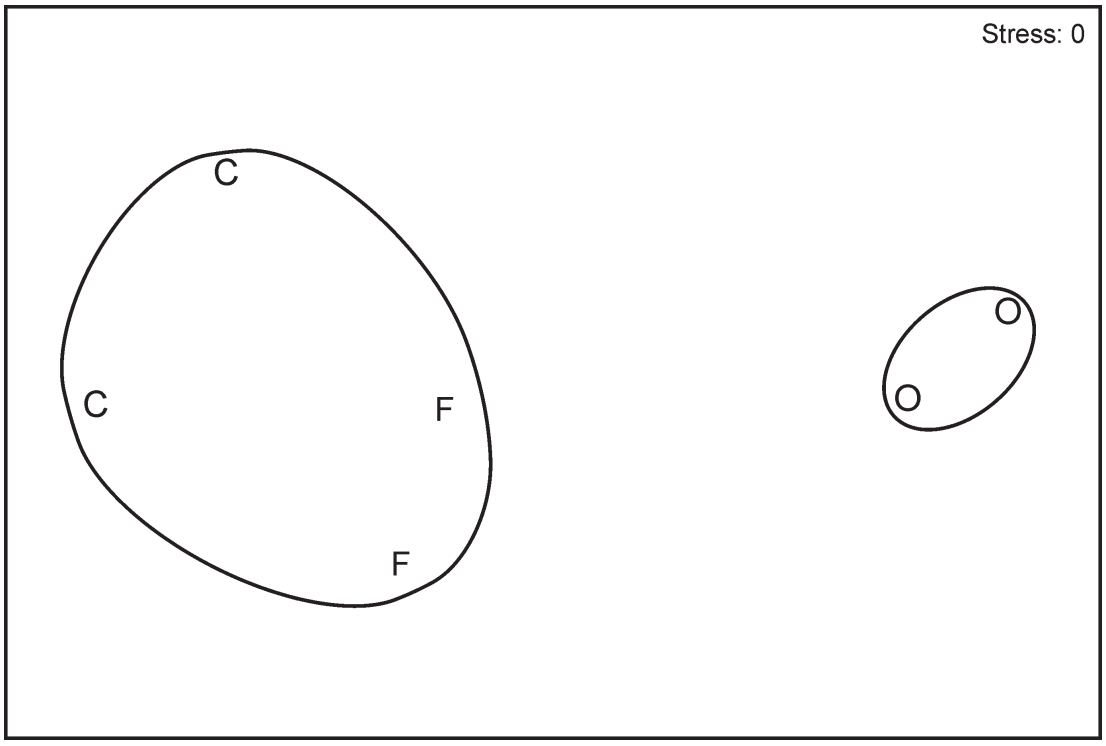

Figure 2. Non-parametric multi-dimensional scaling plot of wild fish community abundances at each site. O = outside cages (fish directly associates to the net-cages), F = Fraile Island, C = Cape Cope. Replicates of each site represent data obtained by divers. Solid lines show two groups according to a cluster classification analysis (70% similarity).

In site Inside cages (I) were collected only European sea bass and gilthead sea bream because these are the cultured species. In the other sites, collected fish species may differ due to their presence in those areas or because they were not

feasible to be captured. Table 1 shows the total length ± SD of each collected species by site.

Species	Sites			
	I	O	F	C
Sparus aurata	20.0±1.7	32.3±2.9		
Dicentrarchus labrax	33.5±11.9	55.7±4.0	49.7±9.9	
Oblada melanoura		20.3±1.2	23.0±4.0	14.3±2.5
Boops boops		29.7±1.2	14.0±1.4	
Mugil cephalus		58.3±3.2		59.3±13.6
Sarpa salpa		40.3±3.9	17.0±1.0	17.5±1.7
Diplodus sargus		28.0±1.4	20.5±2.1	18.0±6.2
Diplodus puntazzo		25.5±2.1	21.0±4.4	20.0±6.6
Diplodus vulgaris		26.5±0.7	17.3±2.3	20.5±2.9
Symphodus tinca			25.8±0.5	23.0±1.7

I = inside cages
O = outside cages
F = Fraile Island
C = Cape Cope

Table 1. Total length mean (± SD, n=4) of captured species in each site.

Stable Isotopes

The concentrations of stable isotopes of the fish feed for the $\delta^{13}C$ were -22.5±0.68 and for $\delta^{15}N$ 7.3±0.65. Differences of $\delta^{13}C$ and $\delta^{15}N$ mean values between white muscle of farmed fish and fish feed were of $\delta^{13}C$ 2.8 and $\delta^{15}N$ 3.0 in *S. aurata*, and $\delta^{13}C$ 3.6 and $\delta^{15}N$ 4.1 in *D. labrax*, that can be considered the normal enrichment for this species in captivity.

The data of accumulation of C and N stable isotopes in the analyzed species (Fig 3) showed that the $\delta^{15}N$ concentration was relatively constant, excepting for *Sarpa salpa* whose concentration declined in sites F and C comparing to site O. The $\delta^{13}C$ isotope concentration was generally more enriched in the sites located further from the fish farm. It also shown that the SD of fishes in sites F and C were generally wider than the results of sites O and I, which SD were shrink.

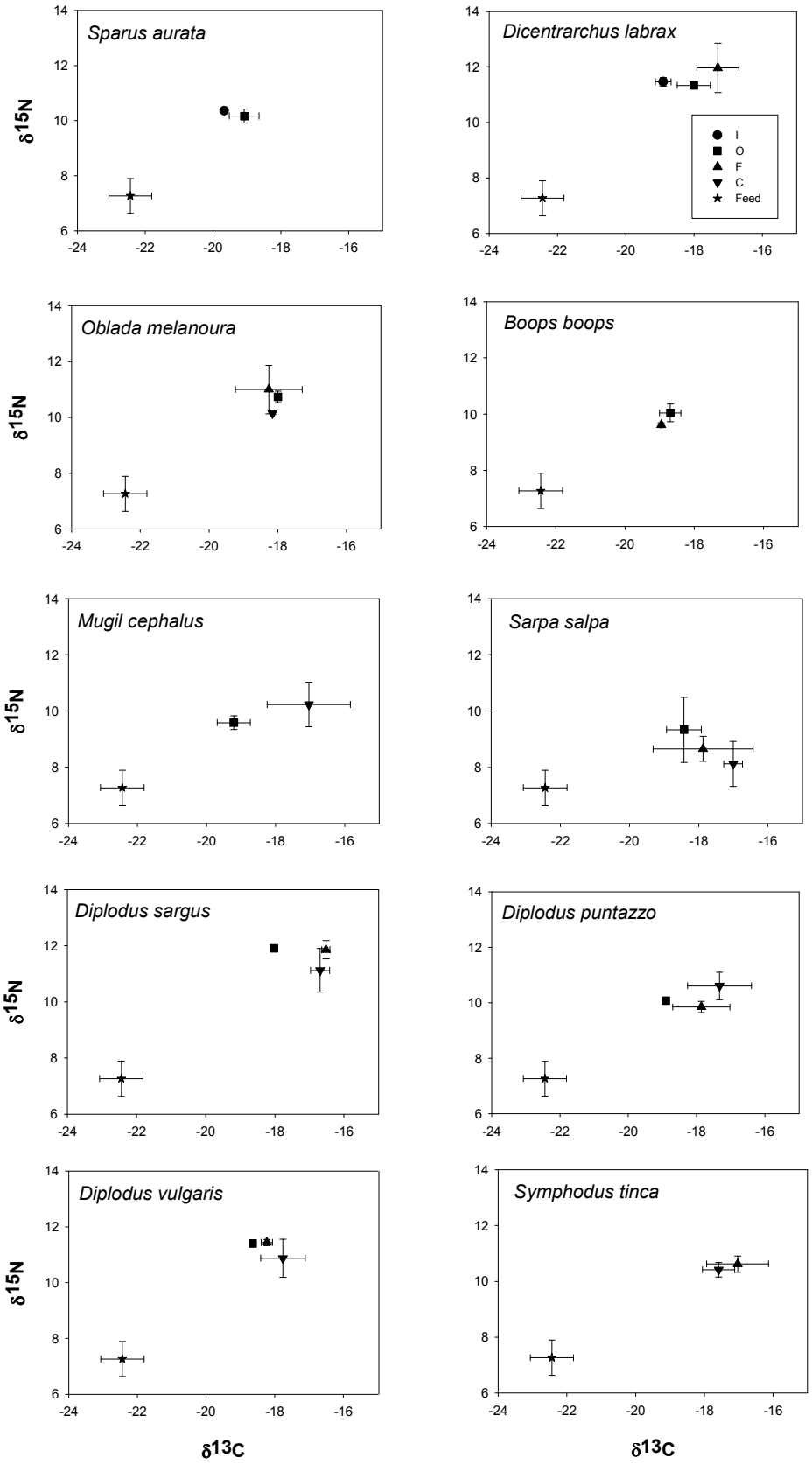

Figure 3. Bi-dimensional plots of $\delta^{13}C$ and $\delta^{15}N$ variation (mean ± SD, n=4) in feed and white muscle of fish by site. (I = inside cages, O = outside cages farm, F = Fraile Island, C = Cape Cope)

The variation of $\delta^{15}N$ in white muscle of analyzed fish was not significant when compared at species level in each site, excepting for a small difference ($p<0.05$) in sites F and C for *Diplodus puntazzo*. While, $\delta^{13}C$ concentration differences were highly significant ($p<0.05$) at specie level between sites for *D. labrax* in site I, and for *Mugil cephalus* and *Diplodus sargus* in site O compared to the rest of the sites where these species were found.

Site	CR	NR	TA	CD	NND	SDNND	C centroid	N centroid
I	1.18	1.40	0.52	0.60	0.10	0.09	-19.14	11.13
O	2.17	2.64	2.80	0.83	0.20	0.14	-18.59	10.59
F	3.38	4.36	8.72	1.36	0.45	0.27	-17.68	10.53
C	2.09	4.33	5.39	1.14	0.24	0.28	-17.37	10.15

I = inside cages
O = outside cages
F = Fraile Island
C = Cape Cope

Table 2 Isotopic metrics of fish communities in each site. $\delta^{13}C$ range (CR), $\delta^{15}N$ range (NR), total area (TA), mean distance to centroid (CD), mean nearest neighbour distance (NND), standard deviation of the nearest neighbour distance (SDNND), spatial position of the centroid of carbon (C centroid) and spatial position of the centroid of nitrogen (N centroid)

Fish community-wide metrics

Results for fish communities were analyzed following the proposed Layman´s metrics (Table 2). In site F we found the widest range for Carbon and Nitrogen as well as the largest total area, mean distance to centroid and the nearest neighbour distance values. The site C present high values of total area but with a minor CR than site F. The minimums values of all Layman´s metrics were presented in site I, which fish were directly influenced by artificial feed. The compression of these metrics in site O, which is more similar to site I, showed a change in the community with respect to the natural populations. The position of the centroid of Nitrogen did not change significantly, but the Carbon centroid evidenced a defined movement to a lower accumulation when the sites are close to the artificial feed of fishes. The

total area of community distribution (Fig. 4) pointed to the widest isotopic niche in site F.

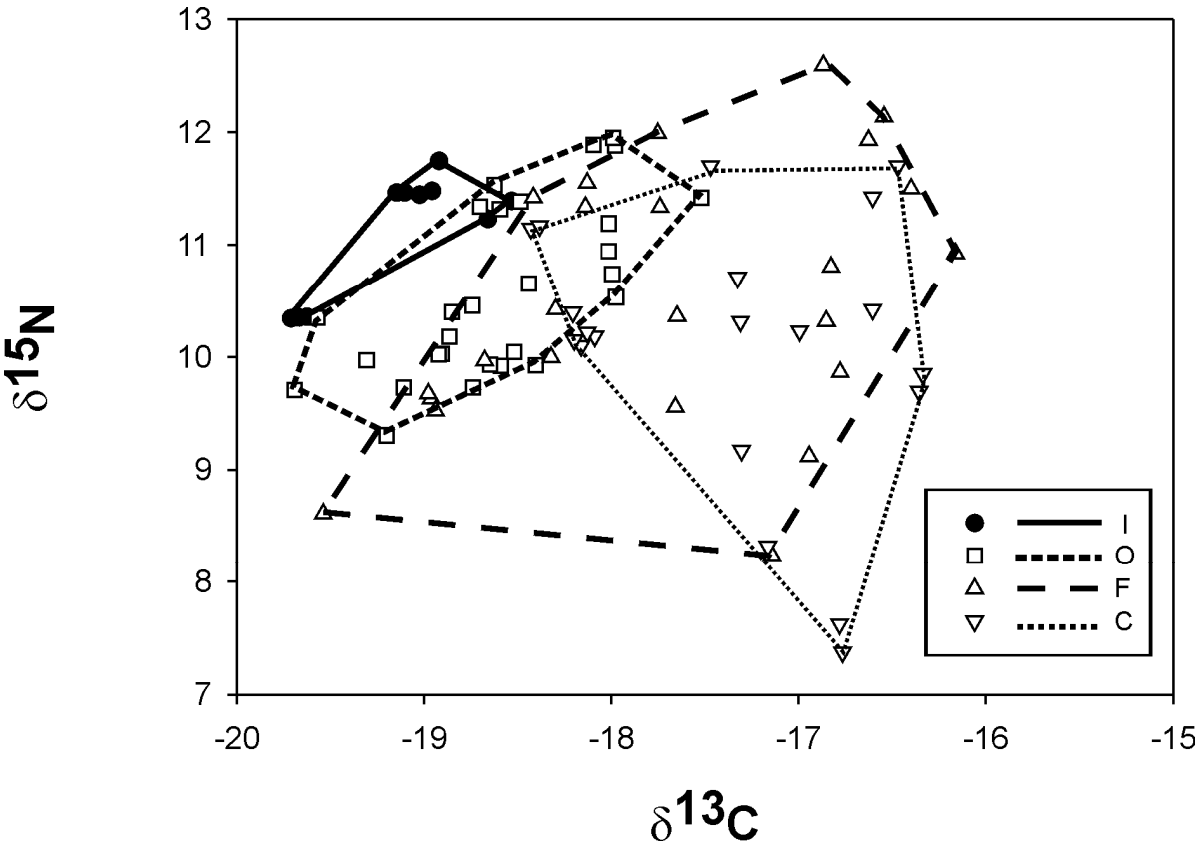

Figure 4. Bi-dimensional plot of $\delta^{13}C$ and $\delta^{15}N$ in fish community at each site. Isotopic width of inside cages (I), outside cages (O), Fraile island (F) and Cope cape (C), is indicated by different lines and defined by total area, the minimum convex polygon bounding most divergent individuals.

DISCUSSION

As Bearhop et al. (2004) pointed, the use of stable isotopes of carbon and nitrogen are one of the most practical and efficient techniques to estimating the trophic niche width in animals. These techniques are commonly used for controlled dietary studies (Pearson *et al.* 2003) and also have success in ecological trophic dynamics (Darimont, Paquet & Reimchen 2009; Layman *et al.* 2007b; Lloret & Marin 2009). In

this case, the analysis of ecological niches in communities of fish could allow a more accurate assessment of the impact level of one of the most important, widespread and growing human activity in Mediterranean coasts.

Our results clearly demonstrate that wild fish associated to fish farm modify their trophic behavior by changing and compressing their trophic niche. This effect would result from their association with marine fish farms and the consumption, either direct or indirect of the formulated feed, which has a different carbon and nitrogen stable isotope signature than the natural food sources in the area. These findings are, to the best of our knowledge, the first approach to the analysis of the trophic status of associated wild fish and the alteration of their normal feeding behavior.

The notorious differences in fish abundance between site O and sites F and C (Fig. 2), concurs with other works reporting wild fish aggregation related to fish farms (Dempster *et al.* 2004; Valle *et al.* 2007). In addition, these results confirm that the farm in our study attract an unusual abundance of fish, pointing to the importance of these aggregations for the environment close to the farm. Dempster et al. (2002) note that these fish assemblages occurs usually in the surroundings of the farms, decreasing dramatically at 200 m from the farm. In our study, site F located at 1200 m from the farm have different abundance levels than for site O, even when we analyze the lengths of species like *Boops boops* or *S. salpa*, which has completely different body sizes, but when we analyze the stable isotopic concentrations, it was found a small overlapping between sites F and O. These results suggest a small indirect exportation to the isotopic carbon of fish feed from where some fish could be using part of the leak of organic matter. Despite even with the high mobility of fish, the communities appear to be not related between them. Site C does not show any relation with the farm.

The different accumulation of stable isotopes between the cultured fish species (*S. aurata* and *D. labrax*) denotes a specific enrichment, which could be due to their particular physiological processes for bioaccumulation. The samples of sites O, F and C present similar nitrogen isotopic levels, probably because those fish does not

change their trophic level. However, from carbon isotopic levels it is feasible to appreciate a progressive accumulation in fish located further the farm. This profile is applicable to the most of the analyzed species, indicate a change in the source of carbon. A minor or "terrestrial" accumulation is related to the feed of cultured fish (the formulated feed is made with some terrestrial ingredients), while the wild fish of sites C or F, which have access to a natural or "marine" source, accumulate more stable isotopic carbon (Schoeninger & Deniro 1984). This data are in agree with stable isotopic studies of Serrano et al (Serrano, Blanes & Orero 2007; Serrano, Barreda & Blanes 2008) realized with *S. aurata* in cultured populations compared to wild fish which are not influenced by aquaculture.

We performed an isotopic total area graph to improve fish community representation based on the trophic behaviour (Fig 4) and considering total area as the minimum convex polygon bounding the most divergent individuals (Layman *et al.* 2007a). In this case, we can consider that the amplitude of 5.39 in the non impacted area (site C) is a normal niche of the fish community, while the biggest amplitude in site F (8.72) is probably because there is not only one group, as the niche width of natural community showed a slight overlapping with the fish associated to the cages, which suggest an small impact of the farm. Interestingly, the area of site O (2.80) presents a clearly compression of niche, despite the fact this group is the most numerous in species, showing the influence of artificial food on the changes in the normal trophic behaviour of these fish. The amplitude areas in sites C and F shows how the heterogeneity of resources is traduced in high isotopic variation and amplitude in the niche width (Darimont, Paquet & Reimchen 2009). The clearly area compression of fish community in site O, even this group has the high number of analyzed species, shows the niche collapse probably debt to the change in feeding behaviour and the decline of variability in the ingest sources of this fish group.

The nitrogen range is different comparing sites I and O with sites F and C (Table 2), supporting our hypothesis of alteration in the trophic behaviour, since it is well known that fish in natural environments feed from different levels, but cultured fish

and farm associated fish have a limited nitrogen range highly dependent of the artificial feed. The CD and SDNND are also related with NR and TA that corroborate these alterations in farm associated fish. The position of N centroid is maintained between sites, but C centroid position show a community change and spatial movement to the more enrichment δ13C area as much as the site is less influenced by the aquaculture; this kind of spatial movement is according with related works about isotopic sources analysis (Darimont, Paquet & Reimchen 2009; Lloret & Marin 2009). Layman et al. (2007b) present an example of niche width collapse by a fragmentation of aquatic environments with the consequent reduction of prey diversity. In our study the niche width collapse is not due to a reduction of resources but is caused by a increase of allocthonous organic material from fish farm which induces a change in wild fish feeding regime. The ecological consequences there are not only the limitation in the natural pathways of energy flow and web architecture; this kind of domestication could become in more susceptible fish communities and in the reduction of the natural response to environmental changes (Tilman *et al.* 1994).

The prohibition of fishing around the 120 m around the considered fish farm (similar than the most of fish farms concessions) help to maintain a stable and constant population of associated wild fish, which are usually bigger than the other from areas not influenced (Table 1). This is in agreement with the suggestion of these areas as small Marine Protected Areas (MPAs) that can act like sanctuaries for pelagic species, whose fish stocks can contribute to the growth of the natural fish stocks (Dempster *et al.* 2002). That may be have deeply implications since it could benefit the local fisheries, reduce the uneaten feed and other particular organic load to the benthos, and maintain the local ichthyic diversity; but also could bring some problems due to the accumulation of fish in an area not prepared for that biomass, which can also consume local natural small resources, could facilitate a disease transfer from cultured fishes to "real" wild animals and may increase the risk of genetic contamination to wild natural stocks (Crozier 2000; Sepulveda, Marin & Carvajal 2004).

Summarizing, in this study we demonstrate that fish farms and their leaks of organic matter alter the fish trophic behaviour by collapsing the niche width of the wild fish associated to this aquaculture activity. The consequences derived of this change in important and big communities of pelagic fishes could bring environmental alterations which have to be deeply evaluated. Nevertheless, this study provides us initial information of the trophic status in farm associated wild fish, which will be enhanced in the future studies to develop and design management strategies for this important resource in Mediterranean ecosystems.

ACKNOWLEDGMENTS

We thank to Culmarex S.A. by the permit to access to their fish farm during this study. To Julián Rivas for his invaluable help in the fish capture, Cayetano Gutierrez-Cánovas and Javier Lloret for their assistance with the metrics analysis, Patricia Castillo-Briceño for her useful comments to the manuscript, Pedro Abellán for the map, and CAID service from University of Murcia for drying the samples. This work was supported by the Programme AlBan - the European Union Programme of High Level Scolarships for Latin America (FN-M scholarship No. E07D401236EC) and from the Spanish Ministry of Education and Science (CS-L Grant)

References

Alarcon,J.A., Magoulas,A., Georgakopoulos,T., Zouros,E. & Alvarez,M.C. (2004) Genetic comparison of wild and cultivated European populations of the gilthead sea bream (Sparus aurata). *Aquaculture*, 230**,** 65-80.

Bearhop,S., Adams,C.E., Waldron,S., Fuller,R.A. & Macleod,H. (2004) Determining trophic niche width: a novel approach using stable isotope analysis. *Journal of Animal Ecology*, 73**,** 1007-1012.

Bell,J.G., Preston,T., Henderson,R.J., Strachan,F., Bron,J.E., Cooper,K. & Morrison,D.J. (2007) Discrimination of wild and cultured european sea bass (Dicentrarchus labrax) using chemical and isotopic analyses. *Journal of Agricultural and Food Chemistry*, 55**,** 5934-5941.

Bjordal,A.A. & Skar,A.B. (1992) Tagging of saithe (*Pollachious virens* L.) at a Norwegian fish farm: Preliminary results of migration. *ICES Demersal Fish committee* (ed A.Other (ed.)), pp. 67-87. International Council for the Exploration of the Sea, Copenhagen, Denmark.

Boyra,A., Sanchez-Jerez,P., Tuya,F., Espino,F. & Haroun,R. (2004) Attraction of wild coastal fishes to an Atlantic subtropical cage fish farms, Gran Canaria, Canary Islands. *Environmental Biology of Fishes*, 70**,** 393-401.

Bricout,J. (1982) Isotope ratios in environmental studies. *International Journal of Mass Spectrometry and Ion Physics*, 45**,** 195-205.

Busetto,M.L., Moretti,V.M., Moreno-Rojas,J.M., Caprino,F., Giani,I., Malandra,R., Bellagamba,F. & Guillou,C. (2008) Authentication of farmed and wild turbot (Psetta maxima) by fatty acid and isotopic analyses combined with chemometrics. *Journal of Agricultural and Food Chemistry*, 56**,** 2742-2750.

Carabel,S., Godinez-Dominguez,E., Verisimo,P., Fernandez,L. & Freire,J. (2006) An assessment of sample processing methods for stable isotope analyses of marine food webs. *Journal of Experimental Marine Biology and Ecology*, 336**,** 254-261.

Castro,J.J., Santiago,J.A. & Santana-Ortega,A.T. (2001) A general theory on fish aggregation to floating objects: An alternative to the meeting point hypothesis. *Reviews in Fish Biology and Fisheries*, 11**,** 255-277.

Cifuentes,L.A., Sharp,J.H. & Fogel,M.L. (1988) Stable Carbon and Nitrogen Isotope Biogeochemistry in the Delaware Estuary. *Limnology and Oceanography*, 33**,** 1102-1115.

Clarke,K.R. & Warwick,R.M. (1994) Similarity-Based Testing for Community Pattern - the 2-Way Layout with No Replication. *Marine Biology*, 118**,** 167-176.

Crozier,W.W. (2000) Escaped farmed salmon, Salmo salar L., in the Glenarm River, Northern Ireland: genetic status of the wild population 7 years on. *Fisheries Management and Ecology*, 7**,** 437-446.

Darimont,C.T., Paquet,P.C. & Reimchen,T.E. (2009) Landscape heterogeneity and marine subsidy generate extensive intrapopulation niche diversity in a large terrestrial vertebrate. *Journal of Animal Ecology*, 78**,** 126-133.

Dempson,J.B. & Power,M. (2004) Use of stable isotopes to distinguish farmed from wild Atlantic salmon, Salmo salar. *Ecology of Freshwater Fish*, 13**,** 176-184.

Dempster,T., Moe,H., Fredheim,A., Jensen,Q. & Sanchez-Jerez,P. (2007) Escapes of marine fish from sea-cage aquaculture in the Mediterranean Sea: status and prevention. *Impact of mariculture on coastal ecosystems, CIESM Workshop Monographs No. 32* (ed F. Briand), pp. 55-60. Lisboa, 21-24 Feb.

Dempster,T., Sanchez-Jerez,P., Bayle-Sempere,J. & Kingsford,M. (2004) Extensive aggregations of wild fish at coastal sea-cage fish farms. *Hydrobiologia*, 525**,** 245-248.

Dempster,T., Sanchez-Jerez,P., Bayle-Sempere,J.T., Gimenez-Casalduero,F. & Valle,C. (2002) Attraction of wild fish to sea-cage fish farms in the south-western Mediterranean Sea: spatial and short-term temporal variability. *Marine Ecology-Progress Series*, 242**,** 237-252.

Dempster,T., Sanchez-Jerez,P., Uglem,I. & Bjorn,P.A. (2010) Species-specific patterns of aggregation of wild fish around fish farms. *Estuarine Coastal and Shelf Science*, 86**,** 271-275.

Dempster,T., Uglem,I., Sanchez-Jerez,P., Fernandez-Jover,D., Bayle-Sempere,J., Nilsen,R. & Bjorn,P.A. (2009) Coastal salmon farms attract large and persistent aggregations of wild fish: an ecosystem effect. *Marine Ecology-Progress Series*, 385**,** 1-14.

Deudero,S., Pinnegar,J.K., Polunin,N.V.C., Morey,G. & Morales-Nin,B. (2004) Spatial variation and ontogenic shifts in the isotopic composition of Mediterranean littoral fishes. *Marine Biology*, 145**,** 971-981.

FAO. The state of World Fisheries and Aquaculture. 2006. Rome, United Nations. Food and Agricultural Organization.
Ref Type: Edited Book

Fernandez-Jover,D., Sanchez-Jerez,P., Bayle-Sempere,J., Carratala,A. & Leon,V.M. (2007) Addition of dissolved nitrogen and dissolved organic carbon from wild fish faeces and food around Mediterranean fish farms: Implications for waste-dispersal models. *Journal of Experimental Marine Biology and Ecology*, 340**,** 160-168.

Freon,P. & Dagorn,L. (2000) Review of fish associative behaviour: toward a generalisation of the meeting point hypothesis. *Reviews in Fish Biology and Fisheries*, 10**,** 183-207.

Fry,B., Cieri,M., Hughes,J., Tobias,C., Deegan,L.A. & Peterson,B. (2008) Stable isotope monitoring of benthic-planktonic coupling using salt marsh fish. *Marine Ecology-Progress Series*, 369**,** 193-204.

Grey,J. (2001) Ontogeny and dietary specialization in brown trout (Salmo trutta L.) from Loch Ness, Scotland, examined using stable isotopes of carbon and nitrogen. *Ecology of Freshwater Fish*, 10**,** 168-176.

Grigorakis,K., Taylor,K.D.A. & Alexis,M.N. (2003) Organoleptic and volatile aroma compounds comparison of wild and cultured gilthead sea bream (Sparus aurata): sensory differences and possible chemical basis. *Aquaculture*, 225**,** 109-119.

Harmelin-Vivien,M., Harmelin,J., Chauvet,C., Duval,C. & and 7 others (1985) Evaluation visuelle des peuplements et populations de poissons. Methodes et problemes. *Revue d'Ecologie*, 40**,** 467-539.

Hobson,K.A. & Welch,H.E. (1992) Determination of Trophic Relationships Within A High Arctic Marine Food Web Using Delta-C-13 and Delta-N-15 Analysis. *Marine Ecology-Progress Series*, 84**,** 9-18.

Hunter,J.R. & Mitchell,C.T. (1968) Field Experiments on Attraction of Pelagic Fish to Floating Objects. *Journal du Conseil*, 31**,** 427-435.

ICES (2006) *Report of the Working Group on Environmental Interactions of Mariculture (WGEIM)*. Narragansett, Rhode Island, USA.

Jennings,S., Renones,O., MoralesNin,B., Polunin,N.V.C., Moranta,J. & Coll,J. (1997) Spatial variation in the N-15 and C-13 stable isotope composition of plants, invertebrates and fishes on Mediterranean reefs: Implications for the study of trophic pathways. *Marine Ecology-Progress Series*, 146**,** 109-116.

Kurtovic,B., Teskeredzic,E. & Teskeredzic,Z. (2008) Histological comparison of spleen and kidney tissue from farmed and wild European sea bass (Dicentrarchus labrax L.). *Acta Adriatica*, 49**,** 147-154.

Layman,C.A., Arrington,D.A., Montana,C.G. & Post,D.M. (2007a) Can stable isotope ratios provide for community-wide measures of trophic structure? *Ecology*, 88**,** 42-48.

Layman,C.A., Quattrochi,J.P., Peyer,C.M. & Allgeier,J.E. (2007b) Niche width collapse in a resilient top predator following ecosystem fragmentation. *Ecology Letters*, 10**,** 937-944.

Lefebvre,S., Harma,C. & Blin,J.L. (2009) Trophic typology of coastal ecosystems based on delta C-13 and delta N-15 ratios in an opportunistic suspension feeder. *Marine Ecology-Progress Series*, 390**,** 27-37.

Lloret,J. & Marin,A. (2009) The role of benthic macrophytes and their associated macroinvertebrate community in coastal lagoon resistance to eutrophication. *Marine Pollution Bulletin*, 58**,** 1827-1834.

Machias,A., Karakassis,I., Labropoulou,M., Somarakis,S., Papadopoulou,K.N. & Papaconstantinou,C. (2004) Changes in wild fish assemblages after the establishment of a fish farming zone in an oligotrophic marine ecosystem. *Estuarine Coastal and Shelf Science*, 60**,** 771-779.

Marin-Guirao,L., Llotet,J. & Marin,A. (2008) Carbon and nitrogen stable isotopes and metal concentration in food webs from a mining-impacted coastal lagoon. *Science of the Total Environment*, 393**,** 118-130.

Maruyama,A., Yamada,Y., Rusuwa,B. & Yuma,M. (2001) Change in stable nitrogen isotope ratio in the muscle tissue of a migratory goby, Rhinogobius sp., in a natural setting. *Canadian Journal of Fisheries and Aquatic Sciences*, 58**,** 2125-2128.

Moreno-Rojas,J.M., Serra,F., Giani,I., Moretti,V.M., Reniero,F. & Guillou,C. (2007) The use of stable isotope ratio analyses to discriminate wild and farmed gilthead sea bream (*Sparus aurata*). *RAPID COMMUNICATIONS IN MASS SPECTROMETRY*, 21**,** 207-211.

Pearson,S.F., Levey,D.J., Greenberg,C.H. & del Rio,C.M. (2003) Effects of elemental composition on the incorporation of dietary nitrogen and carbon isotopic signatures in an omnivorous songbird. *Oecologia*, 135**,** 516-523.

Periago,M.J., Ayala,M.D., Lopez-Albors,O., Abdel,I., Martinez,C., Garcia-Alcazar,A., Ros,G. & Gil,F. (2005) Muscle cellularity and flesh quality of wild and fanned sea bass, Dicentrarchus labrax L. *Aquaculture*, 249**,** 175-188.

Pinnegar,J.K. & Polunin,N.V.C. (1999) Differential fractionation of delta C-13 and delta N-15 among fish tissues: implications for the study of trophic interactions. *Functional Ecology*, 13**,** 225-231.

Pinnegar,J.K. & Polunin,N.V.C. (2000) Contributions of stable-isotope data to elucidating food webs of Mediterranean rocky littoral fishes. *Oecologia*, 122**,** 399-409.

Sanz-Lázaro,C. (2010) *Environmental effects of aquaculture and oil spills*. LAP Lambert Academic Publishing, Saarbrücken, Germany.

Schoeninger,M.J. & Deniro,M.J. (1984) Nitrogen and Carbon Isotopic Composition of Bone-Collagen from Marine and Terrestrial Animals. *Geochimica et Cosmochimica Acta*, 48**,** 625-639.

Sepulveda,F., Marin,S.L. & Carvajal,J. (2004) Metazoan parasites in wild fish and farmed salmon from aquaculture sites in southern Chile. *Aquaculture*, 235**,** 89-100.

Serrano,R., Barreda,M. & Blanes,M.A. (2008) Investigating the presence of organochlorine pesticides and polychlorinated biphenyls in wild and farmed gilthead sea bream (Sparus aurata) from the Western Mediterranean sea. *Marine Pollution Bulletin*, 56**,** 963-972.

Serrano,R., Blanes,M.A. & Orero,L. (2007) Stable isotope determination in wild and farmed gilthead sea bream (Sparus aurata) tissues from the western Mediterranean. *Chemosphere*, 69**,** 1075-1080.

Sudirman, Halide,H., Jompa,J., Iswahyudin,Z. & McKinnon,A.D. (2009) Wild fish associated with tropical sea cage aquaculture in South Sulawesi, Indonesia. *Aquaculture*, 286**,** 233-239.

Thornton,S.F. & Mcmanus,J. (1994) Application of Organic-Carbon and Nitrogen Stable-Isotope and C/N Ratios As Source Indicators of Organic-Matter

Provenance in Estuarine Systems - Evidence from the Tay Estuary, Scotland. *Estuarine Coastal and Shelf Science*, 38**,** 219-233.

Tilman,D., May,R.M., Lehman,C.L. & Nowak,M.A. (1994) Habitat Destruction and the Extinction Debt. *Nature*, 371**,** 65-66.

Uglem,I., Dempster,T., Bjorn,P.A., Sanchez-Jerez,P. & Okland,F. (2009) High connectivity of salmon farms revealed by aggregation, residence and repeated movements of wild fish among farms. *Marine Ecology-Progress Series*, 384**,** 251-260.

Valle,C., Bayle-Sempere,J.T., Dempster,T., Sanchez-Jerez,P. & Gimenez-Casalduero,F. (2007) Temporal variability of wild fish assemblages associated with a sea-cage fish farm in the south-western Mediterranean Sea. *Estuarine Coastal and Shelf Science*, 72**,** 299-307.

Vita,R., Marin,A., Madrid,J.A., Jimenez-Brinquis,B., Cesar,A. & Marin-Guirao,L. (2004) Effects of wild fishes on waste exportation from a Mediterranean fish farm. *Marine Ecology-Progress Series*, 277**,** 253-261.

4. TROPHIC STATUS AND EXPRESSION OF GENES RELATED TO GROWTH, HEALTH AND STRESS STATUS IN EUROPEAN SEA BASS (*Dicentrarchus labrax*). AN ECOLOGICAL APPROACH RELATED TO AQUACULTURE IN OPEN SEA

Trophic status and expression of genes related to growth, health and stress status in European sea bass (*Dicentrarchus labrax*). An ecological approach related to aquaculture in open sea.

Francisco Navarrete-Mier, Department Ecology and Hydrology, University of Murcia. Murcia, 30100, Spain.

Patricia Castillo-Briceño, Department Cell Biology and Histology, University of Murcia. Murcia, 30100, Spain.

Carlos Sanz-Lázaro, Department Ecology and Hydrology, University of Murcia. Murcia, 30100, Spain.

Chiara Piccinetti, Department Marine Sciences, Università Politecnica delle Marche, Ancona, 60131, Italy.

Ander Montoya, Department Animal Physiology, University of Murcia. Murcia, 30100, Spain.

Oliana Carnevali, Department Marine Sciences, Università Politecnica delle Marche, Ancona, 60131, Italy.

Arnaldo Marín, Department Ecology and Hydrology, University of Murcia. Murcia, 30100, Spain.

Abstract

A great amount of large sea bass are associates to marine fish farms and contribute to the natural stock population. Therefore, the knowledge about the individual physiological status of different population of European sea bass could permit the development of management strategies of this very ecological and economically important resource, since the natural stocks are endangered. In this study, the trophic niche status an expression of genes related with growth, stress and health status were analyzed in four fish populations. We evaluate fish different "natural" conditions using trophic analysis of stable isotopes and expression of selected marker genes (IGF-I, IGFII, MSTN, GR, HSP70, IL-1β and TNFα). The results show that farmed fish present a compressed trophic niche with more tending to terrestrial sources, while the molecular approach suggest a gene balance tending to growth and immune activation compared to wild fish. Wild fish presents widest trophic niche as well as the low levels of expression and small variability of the immune-related genes. In addition, farm associated wild fish show an intermediate terrestrial-marine level, however their gene expression profile only present an intermediate level for Il-1β, which then appear as a good biomarker for health status in relation to environmental conditions. In this site we also found higher expression of IGF I, IGF II, MSTN and GR, since all these genes are involved in the body mass- and metabolic- related processes suggesting an enhanced physiological activity. This is in the best of our knowledge the first ecological study in an integrate approach for trophic and molecular characterization of fish populations in relation to environmental conditions and marine aquaculture impact.

Keywords: Wild fish associated, fish farms, IGF, Myostatin, GR, HSP70, IL-1β, TNFα, stable isotopes.

Introduction

The European sea bass, *Dicentrarchus labrax* L. (Moronidae, Perciformes) is naturally found in coastal waters of the eastern North Atlantic Ocean from southern Norway to Morocco and throughout the Mediterranean Sea and the Black Sea (Triantafyllidis 2007), where they are extremely important for fisheries. This specie is also the principal marine fish cultured in south Europe (Picchietti et al. 2009), where is widely cultured in floating cages since three decades ago, even it is believed that the farm standing stock would be larger than the wild populations (ICES 2006). In addition, there is also a representative fish population permanently associated to fish farms, about which it is not clear whether they result from farm escapes (Dempster et al. 2007) or from originally wild stock. However, since fishing activities are banned around the farms, individuals in those fish populations reach larger sizes than coastal populations that are under heavy fishing pressure which is an impediment for achieving optimal breeding conditions (Machias et al. 2004). Thus, the possible contribution of these associated fish to natural stocks deserves a deeper study. In this scenario, European sea bass is a useful and relevant model for ecological interactions research about marine fish populations with economical importance in the Mediterranean area.

Environmental conditions and the adaptability of organisms to that conditions would affect their general fitness (Hofmann et al. 2010), in this sense aquaculture and other anthropogenic activities have an important impact in the development, health status and reproductive success of associated marine organisms. In European sea bass, it still unknown the effect of fish farms on their wild and farm associated populations, but it is plausible to think that this could be related to alterations in their trophic behavior and subsequently in their physiological status.

The use of stable isotopes of carbon and nitrogen has been emerging as reliable tool for elucidate trophic time-integrated structures and inferring pathways of energy flow in food webs (Fry et al. 2008). However, it is still necessary to complement this information from parallel approaches that allow us to evaluate the fish physio-biological conditions that can be modulated in relation to the environment where these fish are leaving and under an ecosystemic approach. In

addition, molecular biology techniques might be an appropriate option, e.g., modern genomic approaches have facilitated the progress in the understanding of the molecular underpinnings of ecological processes (Machado et al. 2009). Particularly, analysis of gene expression in fish constitutes an important tool for the understanding of growth processes as well as in basic physiology studies (Castilho et al. 2009; Douglas, Dawson-Scully & Sokolowski 2005).

Growth is one of the most clearly indicators for the general status in fish, but under natural conditions it is impossible to control all the parameters that can affect the growth, however it is still feasible to analyze it as internal response. Fish growth is strongly related to the growth of muscles and is controlled by some kinds of extrinsic regulators (Peterson, Waldbieser & Bilodeau 2004). The principal genes involved in the fish myogenesis are insuline growth factor-1 (IGF-I), insuline growth factor-2 (IGF-II) and myostatin (MSTN), which are widely used as biomarkers for muscle growth (Ayson et al. 2002; Chauvigne et al. 2003; Duan, Duguay & Plisetskaya 1993). Balance between these genes would show the real growth status of fish. IGF-I and IGF-II are two polypeptides, structurally similar to insulin, that play an important role in the regulation of development and growth by its metabolic and mitogenic action (Reinecke & Collet 1998). MSTN (also named growth and differentiation factor-8 or GDF) is a member of the transforming growth factor β (TGB-β) superfamily and is involved in the regulation of skeletal muscle growth by inhibiting the muscle cell proliferation as opposite to IGFs (McPherron, Lawler & Lee 1997; Rebhan & Funkenstein 2008).

The response to stress is also important to assess the fish welfare. The primary response to stress show an activation of the hipotalamuns-pituitary-interrenal axis which release the cortisol in the blood stream (Mommsen, Vijayan & Moon 1999). In fish the cortisol level in blood is strictly related with the expression of glucocorticoid receptor (GR) (Barton 2002). In regard to the secondary response to stress, there are changes in the expression of heat shock proteins (HSPs), where HSP70 gene is widely studied in fish to assess the stress response to aquaculture conditions (Ackerman & Iwama 2001; Rollo et al. 2006; Smith, Tremblay & Bradley 1999), which

seems to be related to food restriction stress (Cara et al. 2005; Yengkokpam et al. 2008).

It is also known that the immediate environmental conditions may alter immune and inflammatory responses in fish, thus its health status maintaining is also affected (Gomez & Balcazar 2008). Cytokines are glycoproteins that are involved in the extracellular signal mechanisms playing a pivotal role in the balance of host immune response (Ito et al. 2008). Interleukin 1β (IL-1β) and tumoral necrosis factor α (TNFα) are referential cytokines in the assessing of disease signals (Mladineo & Block 2010). IL-1β is a widely studied pleitropic cytokine that plays fundamental roles in innate and acquired defense (Nicola 1994) activating the increase of phagocytosis, lymphocyte proliferation and superoxide production as well as increasing the resistance to Aeromonas spp (Kono & Sakai 2001). TNFα enhances leukocyte migration and phagocyte activity of macrophages, as well as increase the expression of pro-inflammatory cytokines (Saban et al. 2004).

In an approach to evaluate the effect of fish farms on European sea bass, in this study we assess their influence in the general trophic and physiological status of this specie in cultured, farm associated and wild animals. For that purpose, we used (1) trophic analyses with stable isotopes of carbon and nitrogen, and (2) molecular techniques for mRNA expression analysis of selected marker genes (IGFs, MSTN, GR, HSP70, IL-1β and TNFα). The results obtained would be useful to establish a base of knowledge about the welfare status based in the trophic niche and the expression profile of genes related to growth, stress and health condition, in some of the main sea bass populations that can be found in the Mediterranean.

MATERIALS AND METHODS

Study Area. The study was conducted during the winter 2009 in the western Mediterranean. European sea bass were collected from four sites, which were chosen in base to the environmental representatively and the constant presence of fish. Site **A** was located inside cages, where fish are cultured in a marine farm

specialized in gilthead sea bream (*Dicentrarchus labrax*) and European sea bass (*Sparus aurata*), located in Águilas (38°09'40''N, 0°27'26''W). Site **B**, outside cages, where fish are permanently associated to the farm and swims in the surroundings of the same fish farm (ca, 100m around the net-cages), in this area is forbidden all kind of fishery activity. Site **T**, (37°49'15''N, 0°45'03''W) Marine Reserve Tabarca Island, where is feasible to find fish residents in a marine protected area (MPA) with no influence of anthropogenic activities. Site **W**, (37°49'15''N, 0°45'03''W) San Pedro del Pinatar Port, where fish presence is usual and local people fish them by angling, there is no large animals but there are the "normal" sizes available for fishermen in coastal areas. (Fig. 1)

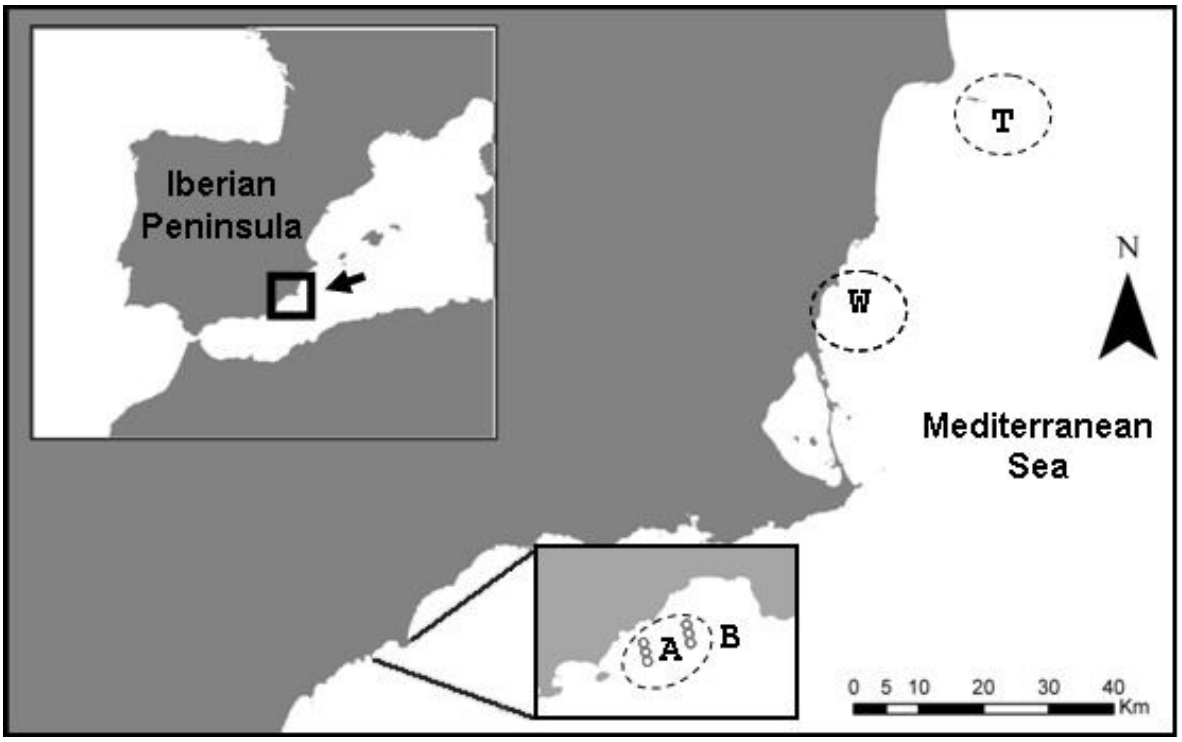

Figure 1. Map of sites of study in the coast of Iberian Peninsula. A (inside cages), B (outside cages), W (San Pedro Pinatar Port), T (Tabarca Island Marine Reserve).

Fish Sampling. There were sampled six males of European sea bass by site, which were obtained by spear-fishing for a minimal environmental impact. Biometrical

data were registered for each fish. Samples of liver, white muscle and spleen were collected for mRNA extraction and immediately frozen in liquid nitrogen to a final storage at -80°C until analysis. Another portion of dorsal white muscle of each fish was collected for stable isotopes analyses (Periago et al. 2005) and stored at -20°C until processing. In addition, samples of different fish feed was obtained from the fish farm, homogenized and analyzed in triplicate to assess the feed sample homogeneity. This study complies with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for using of animals in research.

Stable isotopes analysis. Dorsal white muscle free-bone tissue presents minimum variations in the concentrations of $\delta^{13}C$ and $\delta^{15}N$ (Pinnegar & Polunin 1999), and it is considerate the best tissue in fish for ecological isotopic studies (Deudero et al. 2004; Moreno-Rojas et al. 2007). In this study, muscle and feed samples were dried by liophilization based in Carabel et al (2006). Samples not acidified (Blanco, Deudero & Box 2009) were pulverized to affine powder, homogenized (Grey 2001; Jennings et al. 1997) and subsequently analyzed in duplicate with an elemental analyzer Flash EA1112 (thermoFinnigan) connected to a Delta^{plus} mass spectrometer of isotopic relationships (thermoFinnigan) as previously described (Navarrete-Mier, Sanz-Lazaro & Marin 2010).
All the isotopic data were reported in the conventional δ notation as follows:

$$\delta^{13}C \text{ or } \delta^{15}N = (Rsample / Rstandar - 1)\, 1000\ (‰)$$

Where R represents the 13C/12C or 15N/14N ratio for δ13 C and $\delta^{15}N$, respectively. All $\delta^{13}C$ values were reported as the deviation relative to the Vienna Pee Dee Belemnite Limestone Standart (v-PDB). The $\delta^{15}N$ standards were calibrated and results are reported relative to atmospheric nitrogen.

Molecular analysis

RNA extraction and cDNA synthesis. Total RNA was isolated from tissues using TRI Reagent (Sigma-Aldrich) according to the manufacturer´s protocol. The

concentration and purity of the RNA was estimated by absorbance at 260 nm and $A_{260/280}$ ratio, respectively, using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA quality and integrity was verified on a 1% ethidium bromide-agarose gel. The samples with ratio values ranged from 1.7 to 2.0 were used in reverse transcription PCR (RT-PCR). Total RNA was treated with DNAse (10UI at 37°C for 10 min, MBI Fermentas). A total amount of 1 µg of RNA was used for cDNA first strand synthesis, employing iScript cDNA Synthesis Kit (Bio-Rad). RT-PCR cycling conditions were: 70°C for 5 min, 42°C for 52 min and 72°C for 15 min.

Primer design. Primers were designed using PCR designer software PRIMER3 (http://frodo.wi.mit.edu/primer3/) starting from teleost fish sequences available in GenBank.The primer sequences used in the present study (at final concentration of 10 pmol/µl), are showed in Table 1.

Gene	Accession number	Primer sequence From 5′ to 3′
β-Actin	AY493428	F: GGTACCCATCTCCTGCTCCAA
		R: GAGCGTGGCTACTCCTTCACC
IGF I	AY800248	F: TACAGGCTATGGCCCCAAT
		R: TTGGCAGGTGCACAGTACAT
IGF II	AY839105	F: ACAACAGACGGACCCAGAAC
		R: CGATACTTTTTGGCCCTGAG
MYST	AY839106	F: TTTTGAGCAAACTGCGAATG
		R: CACGTCGTACTGGTCGAGAA
GR	AY549305	F: GCCTTTTGGCATGTACTCAAACC
		R: GGACGACTCTCCATACCTGTTC
HSP70	AY423555	F: ACGGAGAGTCGATTTCGATG
		R: GAAGGACATCAGCGACAACA
IL-1β	AJ269472	F: ACCTCCAACAGCGATCA
		R: AGACTGGCTTTGTCCACCAC
TNFα	DQ070246	F: GACTGGCGAACAACCAGATT
		R: GTCCGCTTCTGTAGCTGTCC

Table 1. Primers used in real-time quantitative RT-PCR

Real time RT-PCR(RT-qPCR). Each sample was analyzed in duplicate RT-qPCR. In base to tissue characteristics we perform the analysis for IGFs, GR and HSP70 in liver; MSTN in muscle; IL1β and TNFα in spleen cDNA (Patruno et al. 2008; Yang et al.

2010). RT-qPCR was optimized (primer annealing temperature and cDNA dilutions) and performed with SYBR green in iQ5 multicolor Real time PCR Detection system (Bio-Rad). The reactions were set on a 96-well plate by mixing 1µl of cDNA diluted at 1:10 (IGFs, GR, HSP70 and TNFα) or 1:5 (MYST and IL1β), 5 µl of 2X concentrated SYBR green PCR master mix (Bio-Rad), 0.3 µM forward primer, and 0.3 µM reverse primer. The thermal profiles for the reaction was: for β-Actin 15min at 95°C and 45 cycles of 20 s at 95°C, 20 s at 58°C, and 20 s at 72°C; for IGF II, GR, HSP70, IL1, TNFα 15min at 95°C and 45 cycles of 20 s at 95°C, 20 s at 60°C, and 20 s at 72°C and for IGF II, MYST 15min at 95°C and 45 cycles of 20 s at 95°C, 20 s at 60°C, and 20 s at 80°C. The fluorescence monitoring occurred at the end of each cycle. Additional dissociation curve analysis was performed and showed in all cases a single melting curve.

Quantification of cDNA. For each mRNA gene expression was normalized to the β-Actin content of each sample using the comparative Cq method ($2^{-\Delta\Delta Cq}$) (Bustin et al 2009) analyzed by the iQ5 optical system software version 2.1 (Bio-Rad). No amplification product was observed in negative control and no primers-dimer formation was observed in the control templates. Modifications of gene expression are represented as fold change relative to the control.

Statistical analysis. Gene expression results were analyzed by one-way ANOVA and the Tukey's test to detect statistically significant differences ($p < 0.05$) using a statistical software package (Prism 5 for Windows version 5.00, Graphpad Software).

Bi-dimensional plots were constructed to represent isotope analysis data of total area populations. The population-wide metrics calculated for each site were used to identify possible differences in trophic diversity and/or trophic redundancy between the sites as described by Lloret & Marín (2009). The six level metric were calculated for all the samples as proposed by Layman et al. (2007a): δ15N range (NR), which shows degree of trophic diversity and vertical food web structure; δ13C range (CR), which show the quantity of basal resources and niche diversification at the base of

the food web; Total area (TA), defined by the minimum convex polygon bounding the most divergent individuals and represent the amount of niche space occupied by a community; Mean distance to centroid (CD) which describe the overall degree of trophic diversity; Mean nearest neighbor distance (NND), which is a proxy of trophic redundancy; Standart deviation of the nearest neighbour distance (SDNND), which indicates the evenness of the distribution of trophic niches in a community. Also we record the C centroid and N centroid, which permit to see the spatial position of the median of the community.

RESULTS:

Fish collection

The mean weight of European sea bass males collected in A site was 1682 g (SD, ± 294), in site B was 2110 g (SD, ± 668), in site T 2203 g (SD, ± 922) and in site W was 358 g (SD, ± 151). There are not significant differences between animals of sites A, B and T, but the weight in site W was significantly lower than in the other sites. However, the fish weights were representative of each site population.

Site	CR	NR	TA	CD	NND	SDNND	C centroid	N centroid
A	1.41	0.42	0.24	0.49	0.27	0.12	-19.14	11.70
B	0.63	0.58	0.17	0.24	0.20	0.16	-17.79	11.69
T	6.09	1.61	2.85	1.42	1.15	1.61	-12.75	12.28
W	0.59	0.70	0.14	0.27	0.20	0.12	-18.78	11.33

Table 2 Isotopic metrics for fish communities in each site o study. $\delta^{13}C$ range (CR), $\delta^{15}N$ range (NR), total area (TA), mean distance to centroid (CD), mean nearest neighbour distance (NND), standard deviation of the nearest neighbour distance (SDNND), spatial position of the centroid of carbon (C centroid) and spatial position of the centroid of nitrogen (N centroid)

Stable isotopes

The concentration of stable isotopes of the fish feed was -22.5 ± 0.68 for the $\delta^{13}C$ and 7.3 ± 0.65 for $\delta^{15}N$, and in both cases were significantly different from the

concentration of the stable isotopes of white muscle in European sea bass from the different sites.

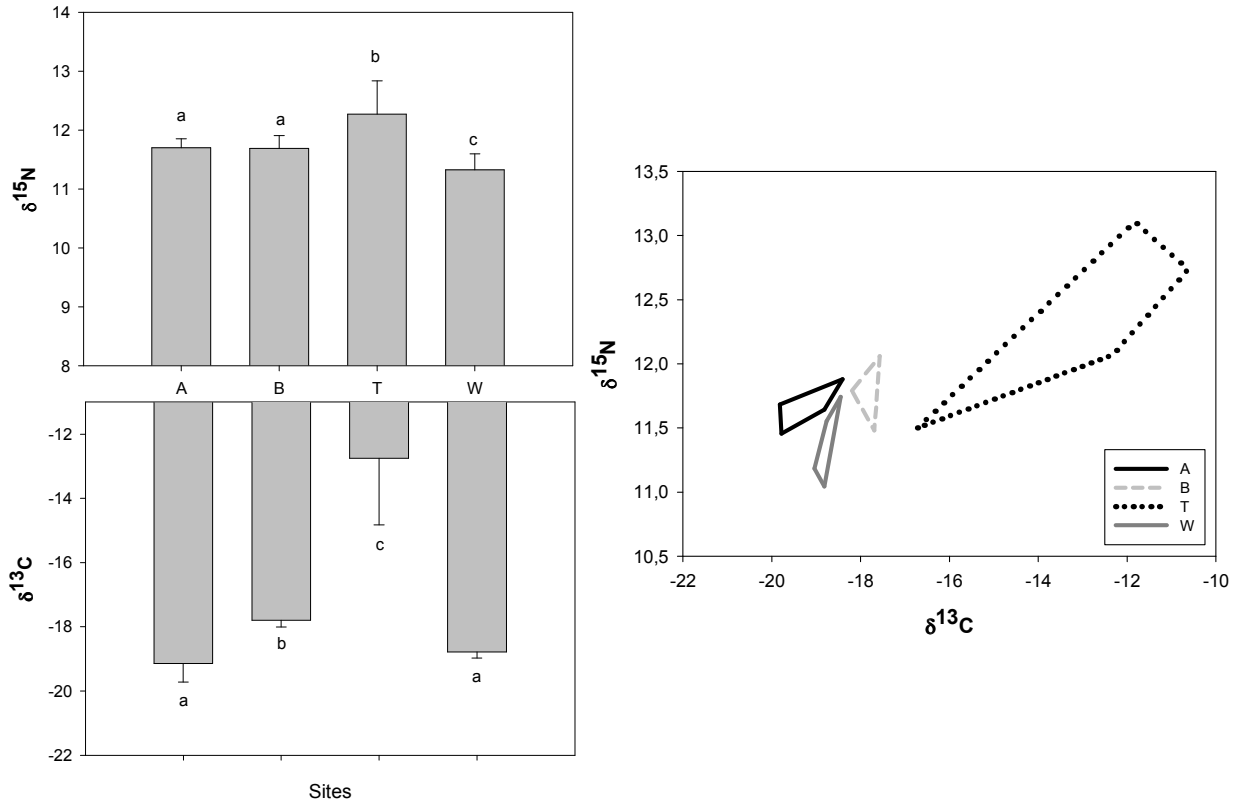

Figure 2. $\delta^{13}C$ and $\delta^{15}N$ in European sea bass (*D. labrax*) populations. a) Stable isotope accumulation by site (mean ± S.D., n= 6). b) Raw isotopic niche widths, defined by total area, the minimum convex polygon bounding most divergent individuals. Different letters denote significant differences between the groups according to Tukey's test for one-way ANOVA ($P < 0.05$)

The data of accumulation of C and N stable isotopes in the analyzed populations are showed in Fig. 2a. The $\delta^{15}N$ concentration in site T is significant higher ($p<0.05$) than in the other sites and the lowest concentration was detected in W site. The highest concentration of $\delta^{13}C$ in fish also corresponds to site T, but the lowest concentrations were detected in sites A and W.

Population-wide metrics

The results of fish populations following the proposed Layman´s metrics (Table 2 and Fig 2b) showed that site T (Tabarca island marine reserve) had the widest range for carbon and nitrogen as well as the largest total area, and that had an influence on the highest numbers of CD, NND, and SDNND detected; then site T was notoriously different from the other sites. In addition, the centroid position of Nitrogen is high in site T, but not far from the centroid of the other sites. With regard to the Carbon centroid the highest value was for site T and the lowest was for site A.

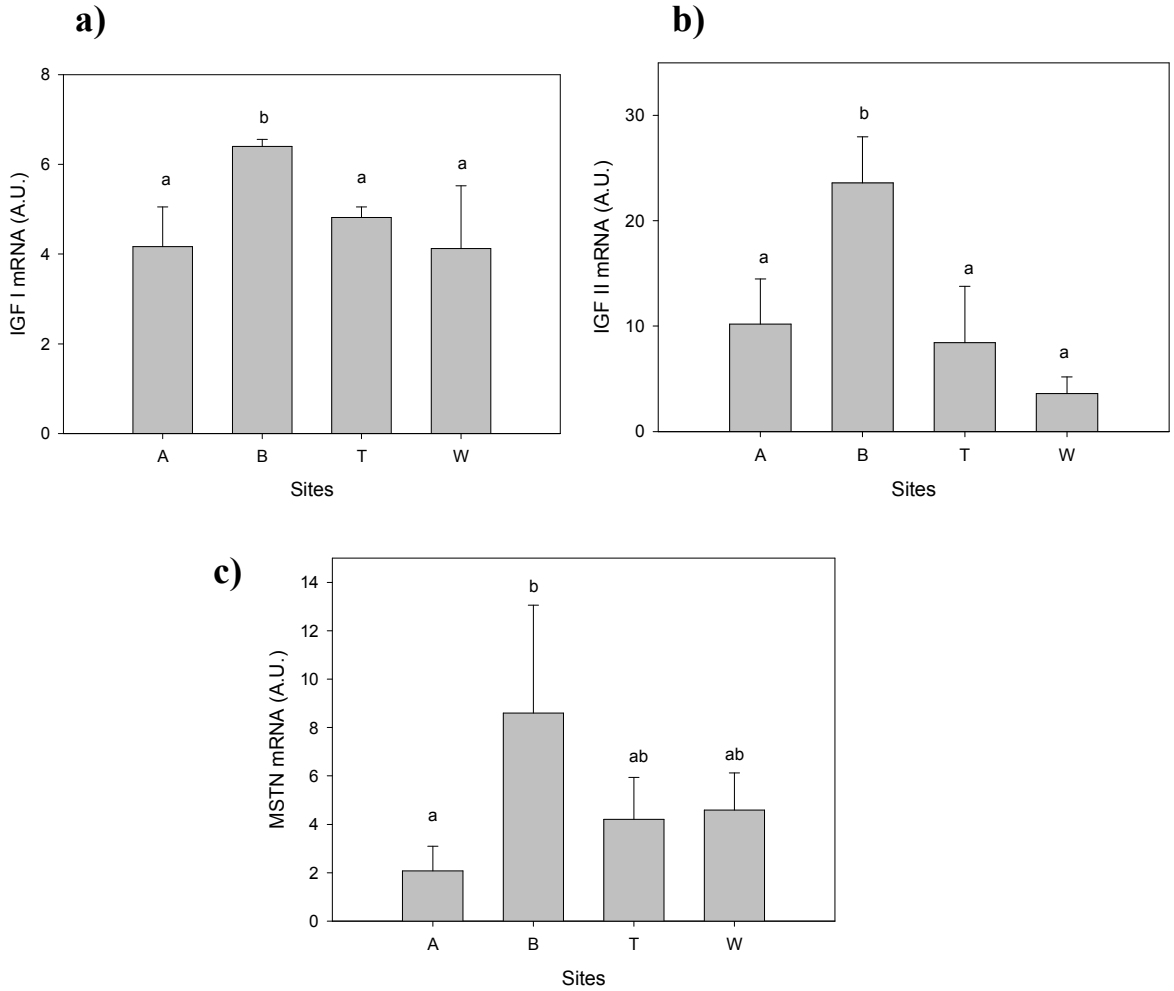

Figure 3. Gene expression profile of growth-related molecules in *D. labrax* by sites. a) IGF-I, b) IGF-II, and c) MSTN. The mRNA levels of the indicated genes were determined by RT-qPCR in amplification products of six fish individually processed. Gene expression is normalized against β-Actin. Each bar represents the mean ± S.D. Different letters denote significant differences between the groups according to Tukey's test for one-way ANOVA ($P < 0.05$).

Molecular approach - mRNA expression analysis by RT-qPCR

The RT-qPCR of growth-related genes showed significant higher levels of IGF-I in site B fish than in the other sites, which were not significantly different between them (Fig. 3a). Similarly, IGF-II showed the highest expression levels in site B fish (Fig. 3b), although the level of expression is in a different scale. Complementary, MSTN expression levels were higher in all the sites compared to site A, but only significantly different in site B (Fig. 3c).

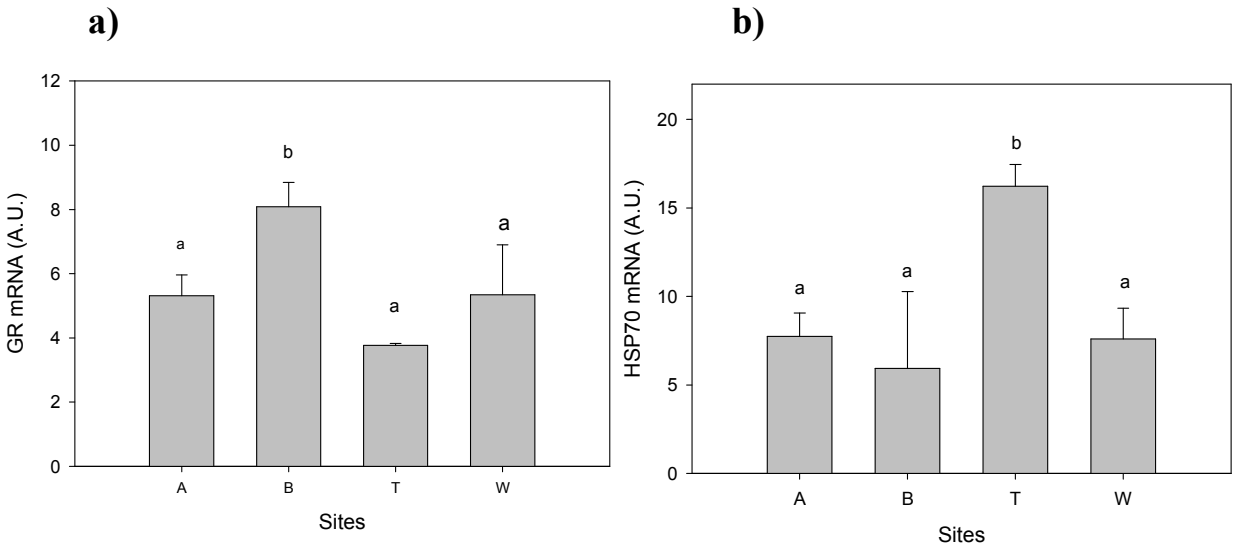

Figure 4. Gene expression profile of stress-related molecules in *D. labrax* by sites. a) GR, b) HSP70. The mRNA levels of the indicated genes were determined by RT-qPCR in amplification products of six fish individually processed. Gene expression is normalized against β-Actin. Each bar represents the mean ± S.D. Different letters denote significant differences between the groups according to Tukey's test for one-way ANOVA ($P < 0.05$).

With regard to the stress-related genes, site B fish had the highest expression of GR with regard to the other sites which were not significantly different between them (Fig. 4a). Concerning HSP70 the significant highest expression was on site T (Fig. 4b).

The expression of health-related genes showed little differences between the sites and no clear dominance of any site for IL-1β neither for TNFα. Wherever, site T fish were in the group of lower expression for both genes (Fig. 5).

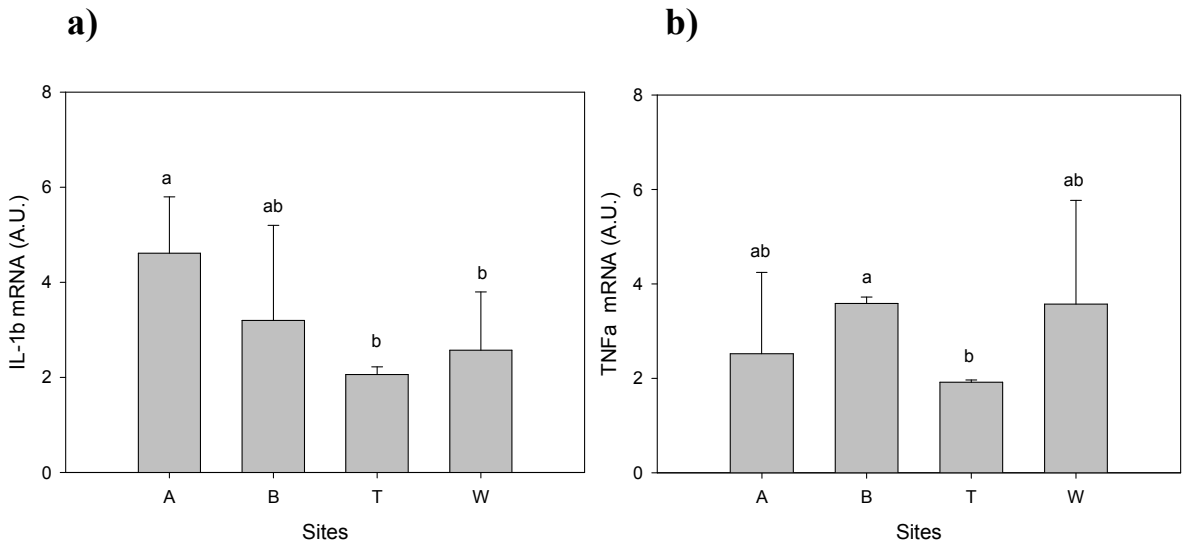

Figure 5. Gene expression profile of health-related molecules in *D. labrax* by sites. a) IL-1β, b) TNFα. The mRNA levels of the indicated genes were determined by RT-qPCR in amplification products of six fish individually processed. Gene expression is normalized against β-Actin. Each bar represents the mean ± S.D. Different letters denote significant differences between the groups according to Tukey's test for one-way ANOVA (P <0.05).

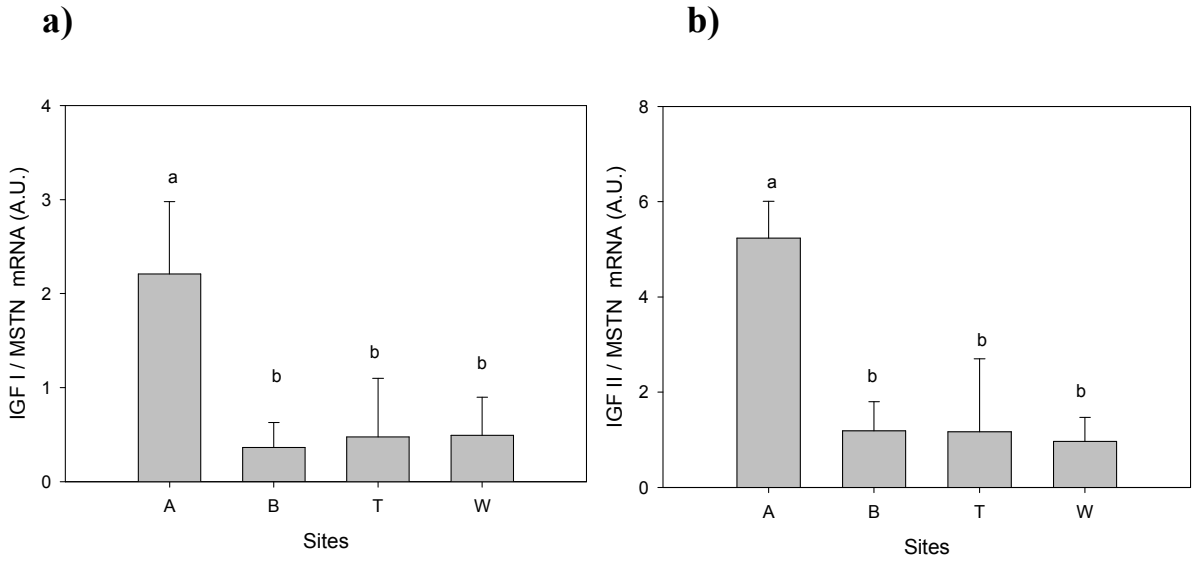

Figure 6. Ratios of gene expression of growth-related molecules in *D. labrax* by sites a) IGF-I / MSTN, b) IGF-II / MSTN. Ratios were calculated from mRNA levels of the indicated genes determined by RT-qPCR. Gene expression is normalized against β-Actin. Each bar represents the mean ± S.D and are representative of six fish. Different letters denote significant differences between the groups according to Tukey's test for one-way ANOVA (P <0.05).

In addition, there were clearly significant higher ratios for IGF-I/MSTN (Fig. 6a) and IGF-II/MSTN (Fig. 6b) in the site A (fish inside cages) with regard to the other sites, whose ratios were not significantly different between them.

DISCUSSION

The results of this study show different niche widths and changes in the expression levels of genes related to growth, stress and health status for the European sea bass populations associated to the fish farm in the Iberian area of Mediterranean. Our results confirm previous studies (Fernandez-Jover et al. 2009) which affirm that fish related to fish farms presents changes in their natural trophic behavior.

Sánchez-Jerez et al (2007) affirm that the constant lost of high protein feed from the farms could become in better body condition of associated fish than their wild counterparts, elsewhere in the sea. This better body condition would imply an increase of spawning success which will contribute to local fisheries. However, the increase of mariculture imply a high risk of genetic contamination of the wild stock by escaped animals, this genetic interaction could bring declines in endemic - evolutionary significant units, and considering that the gene flow is pervasive and persistent it could cause significant decreases in wild/feral sea bass stocks (ICES 2006).

Our results show there is no relation between the weights of fish and their stable isotope accumulation or the expression of genes related to the animal welfare. Furthermore, these parameters appear more related to the characteristics of habitat. The only relation could be in site W between the smallest fish and lower concentration of $\delta^{13}C$, which is logic since small fish consume smaller animals which are in lower levels of the trophic web, wherever that difference was not large. The highest accumulation of $\delta^{15}N$ in site T is normal of a top predator of large size in natural environment areas and which captures all kind of preys to feed. With regard to the accumulation of $\delta^{13}C$, the lowest concentrations sites A and W would be related to their higher influence from terrestrial input, while the highest

accumulation in site T is in agree with the marine feeding regime described for these species in nature (Schoeninger & Deniro 1984).

Site T fish are not only accumulating stable isotopes, but this site also has higher CR, NR, TA, CD, NND and SDNND which means there is a clear variety of resources in their feeding regime, which is the expected in littoral natural areas (Bricout 1982; Pinnegar & Polunin 2000). The bi-plot of niche width (Fig. 2b) show a niche compression (Darimont, Paquet & Reimchen 2009) in sites A, B and T, and the data of carbon centroid position (Table 2) give us a clear vision of the changes and spatial movement due to the artificial source of feed (Layman et al. 2007b). The fish population associated to the farm (B) has a notorious compression of niche related to their wild counterparts (T), indicating a great change in their feeding behavior which is similar to that for cultured fish (A). This feeding regime alteration in a free population which become in efficient reproducers could imply some alterations in the following generations of natural stocks.

With regard to growth related genes in European sea bass populations we could see how the fish of site B have significant higher expression of growth-related genes than fish in the other sites. It is well established that IGF system is mainly involved in growth related to physiological processes such as DNA and protein synthesis (Avella et al. 2007; Patruno et al. 2006; Reinecke & Collet 1998), and also that environmental conditions (temperature, food intake, season) could regulate the expression of these genes (Nordgarden et al. 2006; Pierce et al. 2001). The fish of site B are well feed not only for predating small fish which swim around the net-cages, but also for consuming some of the spill of artificial feed provided to farm fish. Thus, this population has not only a lot of available food but also can swim free, which would be contributing to their better fitness status. The fish of site A have a lot of balanced food but are grown in high density, while the fish in sites T and W have low density conditions but the source of feed is limited. With regard to the MSTN expression profile, the low levels in site A compared to the others appears related to their high density conditions of aquaculture as previously stated for adult zebrafish (*Danio rerio*) in response to overcrowding conditions (Vianello et al. 2003).

In relation to HSP70 mRNA expression profile, it was surprising the highest levels in site T, considering that these proteins has been suggested as biomarkers of environmental quality as they are induced by toxicants even at low concentrations (Lemos et al. 2010). However, it is also stated that other numerous factors can induce HSP expression and stress tolerance, thus their utility as biomarkers of environmental toxins may be limited (Feder & Hofmann 1999). In this case, assuming that natural reserve status of site T implies environmentally healthier conditions than the other sites; we propose that the high levels of HSP70 would be related to other conditions suitable in this type of areas such as the presence of predators as reported for goldfish (*Carassius auratus*) by Kagawa et al. (1999) and/or as part of the hepatic stress response to exercise as described in mammalian models (Gonzalez & Manso 2004). In addition, the increased levels of expression of HSP70 in site T fish would be an advantage for this population according to the described role for HSP family to enhance fish tolerance to upcoming environmental changes, as part of the mechanism where one stressor can induce organisms to better tolerate a subsequent, more severe stressor (Basu et al. 2002). In the case of site B, where apparently are not more stressors than in the other sites, the slightly but significant higher levels of GR in liver could be related to metabolic conditions of this fish which have high amounts of food available and also can swim around without restrains, in the line of the GR role in the processes of lipolysis where its expression is also enhanced by exercising (Campbell et al. 2009).

The Il-1β expression profile results particularly interesting as allows to see a progressive variation from the highest level in farm fish (site A) to the low levels of wild populations (sites T and W), and in the middle term the farm associated fish (site B). Considering that IL-1β is strongly increased in response not only to pathogen but to damage signals in fish (Castillo-Briceno et al. 2009), our results suggest that fish in site T would benefit from healthier environmental conditions, which is also supported by the significant low levels of TNFα too. It is plausible to suppose that these environmental conditions in sites A and B would be altered by the organic wastes from fish farm released in the environment (Karakassis, Pitta & Krom 2005; Sanz-Lazaro & Marin 2006), in agree with the results reviewed by

Johnson et al. (2010) in relation to the effect of the nutrient inputs that can favour opportunistic pathogens, change the density or distribution of suitable hosts/vectors, alter the physical habitat, increases in parasite production, etc.

About IGF I/MSTN and IGF II/MSTN ratios, the notoriously high values in farm fish (site A) suggest a favoured growth tendency in this group compared to all the others, even for farm associated fish (site B), what is expected as farms offer conditions enhanced for growth. It is particularly interesting since the IGF I, IGF II and MSTN levels of expression only allow to detect differences for site B, which appears to be strongly expressing all of these genes. Thus, we can say that for this molecular approach is important to include other parameters related to the context of the analyzed genes as previously stated from another approaches in fish (Castillo-Briceno et al. 2010).

Summarizing, our results mainly show that fish farm present a compressed trophic niche with more tending to terrestrial sources, while the molecular approach suggest a gene balance tending to growth and immune activation compared to wild fish. Related to wild fish, it is remarkable the wide trophic niche in site T as well as the low levels of expression and small variability of the immune-related genes. In addition, farm associated wild fish show, as expected, an intermediate terrestrial-marine level, however their gene expression profile only present an intermediate level for Il-1β, which then appear as a good biomarker for health status in relation to environmental conditions. In this site is also interesting its higher expression of IGF I, IGF II, MSTN and GR since all these genes are involved in the body mass- and metabolic- related processes suggesting an enhanced physiological activity. Whatever the case, this is in the best of our knowledge the first ecological study that use stable isotope and RT-qPCR analyses in an integrate approach for trophic and molecular characterization of fish populations in relation to environmental conditions and marine aquaculture impact.

In that sense, this study is a pioneer in the analysis of ecological status of fish populations with the use of these molecular techniques, and constitutes a base to development future works to expand the vision of ecological research. In addition, the improvement of knowledge about trophic and physiological status of highly important populations of Mediterranean fish such as European sea bass, would permit us the development of accurate management strategies to maintain this resource in equilibrium between the ecosystem and the development of an industry in constant expansion as the aquaculture.

Acknowledgments

We thank to Culmarex S.A. by the permit to access to their fish farm during this study. To Dirección General de Recursos Pesqueros de la Secretaría General de Pesca Marítima (MAPA- Spain) for the access to Tabarca Island Marine Reserve. To CAID and SAI service (University of Murcia) for the facilities in samples treatments. To Julián Rivas and Felio Lozano for his invaluable help for the fish capture. To all the team of Sivilupo e Reproduzione lab (UNIPM). This work was supported by the Programme AlBan - the European Union Programme of High Level Scolarships for Latin America (FN-M scholarship No. E07D401236EC) and from the Spanish Ministry of Education and Science (CS-L Grant)

References

Ackerman,P.A. & Iwama,G.K. (2001) Physiological and cellular stress responses of juvenile rainbow trout to vibriosis. *Journal of Aquatic Animal Health*, 13**,** 173-180.

Avella,M.A., Olivotto,I., Gioacchini,G., Maradonna,F. & Carnevali,O. (2007) The role of fatty acids enrichments in the larviculture of false percula clownfish Amphiprion ocellaris. *Aquaculture*, 273**,** 87-95.

Ayson,F.G., de Jesus,E.G.T., Moriyama,S., Hyodo,S., Funkenstein,B., Gertler,A. & Kawauchi,H. (2002) Differential expression of insulin-like growth factor I and II mRNAs during embryogenesis and early larval development in rabbitfish, Siganus guttatus. *General and Comparative Endocrinology*, 126**,** 165-174.

Barton,B.A. (2002) Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology*, 42**,** 517-525.

Basu,N., Todgham,A.E., Ackerman,P.A., Bibeau,M.R., Nakano,K., Schulte,P.M. & Iwama,G.K. (2002) Heat shock protein genes and their functional significance in fish. *Gene*, 295**,** 173-183.

Blanco,A., Deudero,S. & Box,A. (2009) Muscle and scale isotopic offset of three fish species in the Mediterranean Sea: Dentex dentex, Argyrosomus regius and Xyrichtys novacula. *RAPID COMMUNICATIONS IN MASS SPECTROMETRY*, 23**,** 2321-2328.

Bricout,J. (1982) Isotope ratios in environmental studies. *International Journal of Mass Spectrometry and Ion Physics*, 45**,** 195-205.

Campbell,J.E., Fediuc,S., Hawke,T.J. & Riddell,M.C. (2009) Endurance exercise training increases adipose tissue glucocorticoid exposure: adaptations that facilitate lipolysis. *Metabolism-Clinical and Experimental*, 58**,** 651-660.

Cara,J.B., Aluru,N., Moyano,F.J. & Vijayan,M.M. (2005) Food-deprivation induces HSP70 and HSP90 protein expression in larval gilthead sea bream and rainbow

trout. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 142**,** 426-431.

Carabel,S., Godinez-Dominguez,E., Verisimo,P., Fernandez,L. & Freire,J. (2006) An assessment of sample processing methods for stable isotope analyses of marine food webs. *Journal of Experimental Marine Biology and Ecology*, 336**,** 254-261.

Castilho,P.C., Buckley,B.A., Somero,G. & Block,B.A. (2009) Heterologous hybridization to a complementary DNA microarray reveals the effect of thermal acclimation in the endothermic bluefin tuna (Thunnus orientalis). *Molecular Ecology*, 18**,** 2092-2102.

Castillo-Briceno,P., Arizcun-Arizcun,M., Meseguer,J., Mulero,V. & Garcia-Ayala,A. (2010) Correlated expression profile of extracellular matrix-related molecules during the inflammatory response of the teleost fish gilthead seabream. *Developmental and Comparative Immunology*, 34**,** 1051-1058.

Castillo-Briceno,P., Sepulcre,M.P., Chaves-Pozo,E., Meseguer,J., Garcia-Ayala,A. & Mulero,V. (2009) Collagen regulates the activation of professional phagocytes of the teleost fish gilthead seabream. *Molecular Immunology*, 46**,** 1409-1415.

Chauvigne,F., Gabillard,J.C., Weil,C. & Rescan,P.Y. (2003) Effect of refeeding on IGFI, IGFII, IGF receptors, FGF2, FGF6, and myostatin mRNA expression in rainbow trout myotomal muscle. *General and Comparative Endocrinology*, 132**,** 209-215.

Darimont,C.T., Paquet,P.C. & Reimchen,T.E. (2009) Landscape heterogeneity and marine subsidy generate extensive intrapopulation niche diversity in a large terrestrial vertebrate. *Journal of Animal Ecology*, 78**,** 126-133.

Dempster,T., Moe,H., Fredheim,A., Jensen,Q. & Sanchez-Jerez,P. (2007) Escapes of marine fish from sea-cage aquaculture in the Mediterranean Sea: status and prevention. *Impact of mariculture on coastal ecosystems, CIESM Workshop Monographs No. 32* (ed F. Briand), pp. 55-60. Lisboa, 21-24 Feb.

Deudero,S., Pinnegar,J.K., Polunin,N.V.C., Morey,G. & Morales-Nin,B. (2004) Spatial variation and ontogenic shifts in the isotopic composition of Mediterranean littoral fishes. *Marine Biology*, 145**,** 971-981.

Douglas,S.J., Dawson-Scully,K. & Sokolowski,M.B. (2005) The neurogenetics and evolution of food-related behaviour. *Trends in Neurosciences*, 28**,** 644-652.

Duan,C.M., Duguay,S.J. & Plisetskaya,E.M. (1993) Insulin-Like Growth Factor-I (Igf-I) Messenger-Rna Expression in Coho Salmon, Oncorhynchus-Kisutch - Tissue Distribution and Effects of Growth-Hormone Prolactin Family Proteins. *Fish Physiology and Biochemistry*, 11**,** 371-379.

Feder,M.E. & Hofmann,G.E. (1999) Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annual Review of Physiology*, 61**,** 243-282.

Fernandez-Jover,D., Sanchez-Jerez,P., Bayle-Sempere,J.T., Arechavala-Lopez,P., Martinez-Rubio,L., Jimenez,J.A.L. & Lopez,F.J.M. (2009) Coastal fish farms are settlement sites for juvenile fish. *Marine Environmental Research*, 68**,** 89-96.

Fry,B., Cieri,M., Hughes,J., Tobias,C., Deegan,L.A. & Peterson,B. (2008) Stable isotope monitoring of benthic-planktonic coupling using salt marsh fish. *Marine Ecology-Progress Series*, 369**,** 193-204.

Gomez,G.D. & Balcazar,J.L. (2008) A review on the interactions between gut microbiota and innate immunity of fish. *Fems Immunology and Medical Microbiology*, 52**,** 145-154.

Gonzalez,B. & Manso,R. (2004) Induction, modification and accumulation of HSP70s in the rat liver after acute exercise: early and late responses. *Journal of Physiology-London*, 556**,** 369-385.

Grey,J. (2001) Ontogeny and dietary specialization in brown trout (Salmo trutta L.) from Loch Ness, Scotland, examined using stable isotopes of carbon and nitrogen. *Ecology of Freshwater Fish*, 10**,** 168-176.

Hofmann,C.M., O'Quin,K.E., Smith,A.R. & Carleton,K.L. (2010) Plasticity of opsin gene expression in cichlids from Lake Malawi. *Molecular Ecology*, 19**,** 2064-2074.

ICES (2006) *Report of the Working Group on Environmental Interactions of Mariculture (WGEIM)*. Narragansett, Rhode Island, USA.

Ito,K., Takizawa,F., Yoshiura,Y., Ototake,M. & Nakanishi,T. (2008) Expression profile of cytokine and transcription factor genes during embryonic development of zebrafish Danio rerio. *Fisheries Science*, 74**,** 391-396.

Jennings,S., Renones,O., MoralesNin,B., Polunin,N.V.C., Moranta,J. & Coll,J. (1997) Spatial variation in the N-15 and C-13 stable isotope composition of plants, invertebrates and fishes on Mediterranean reefs: Implications for the study of trophic pathways. *Marine Ecology-Progress Series*, 146**,** 109-116.

Johnson,P.T.J., Townsend,A.R., Cleveland,C.C., Glibert,P.M., Howarth,R.W., McKenzie,V.J., Rejmankova,E. & Ward,M.H. (2010) Linking environmental nutrient enrichment and disease emergence in humans and wildlife. *Ecological Applications*, 20**,** 16-29.

Kagawa,N., Ryo,K. & Mugiya,Y. (1999) Enhanced expression of stress protein 70 in the brains of goldfish, Carassius auratus, reared with bluegills, Lepomis macrochirus. *Fish Physiology and Biochemistry*, 21**,** 103-110.

Karakassis,I., Pitta,P. & Krom,M.D. (2005) Contribution of fish farming to the nutrient loading of the Mediterranean. *Scientia Marina*, 69**,** 313-321.

Kono,T. & Sakai,M. (2001) The analysis of expressed genes in the kidney of Japanese flounder, Paralichthys olivaceus, injected with the immunostimulant peptidoglycan. *Fish & Shellfish Immunology*, 11**,** 357-366.

Layman,C.A., Arrington,D.A., Montana,C.G. & Post,D.M. (2007a) Can stable isotope ratios provide for community-wide measures of trophic structure? *Ecology*, 88**,** 42-48.

Layman,C.A., Quattrochi,J.P., Peyer,C.M. & Allgeier,J.E. (2007b) Niche width collapse in a resilient top predator following ecosystem fragmentation. *Ecology Letters*, 10**,** 937-944.

Lemos,M.F.L., Esteves,A.C., Samyn,B., Timperman,I., van Beeumen,J., Correia,A., van Gestel,C.A.M. & Soares,A.M.V.M. (2010) Protein differential expression induced by endocrine disrupting compounds in a terrestrial isopod. *Chemosphere*, 79**,** 570-576.

Lloret,J. & Marin,A. (2009) The role of benthic macrophytes and their associated macroinvertebrate community in coastal lagoon resistance to eutrophication. *Marine Pollution Bulletin*, 58**,** 1827-1834.

Machado,H.E., Pollen,A.A., Hofmann,H.A. & Renn,S.C. (2009) Interspecific profiling of gene expression informed by comparative genomic hybridization: A review and a novel approach in African cichlid fishes. *Integrative and Comparative Biology*, 49**,** 644-659.

Machias,A., Karakassis,I., Labropoulou,M., Somarakis,S., Papadopoulou,K.N. & Papaconstantinou,C. (2004) Changes in wild fish assemblages after the establishment of a fish farming zone in an oligotrophic marine ecosystem. *Estuarine Coastal and Shelf Science*, 60**,** 771-779.

McPherron,A.C., Lawler,A.M. & Lee,S.J. (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature*, 387**,** 83-90.

Mladineo,I. & Block,B.A. (2010) Expression of cytokines IL-1 beta and TNF-alpha in tissues and cysts surrounding Didymocystis wedli (Digenea, Didymozoidae) in the Pacific bluefin tuna (Thunnus orientalis). *Fish & Shellfish Immunology*, 29**,** 487-493.

Mommsen,T.P., Vijayan,M.M. & Moon,T.W. (1999) Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries*, 9**,** 211-268.

Moreno-Rojas,J.M., Serra,F., Giani,I., Moretti,V.M., Reniero,F. & Guillou,C. (2007) The use of stable isotope ratio analyses to discriminate wild and farmed gilthead sea bream (*Sparus aurata*). *RAPID COMMUNICATIONS IN MASS SPECTROMETRY*, 21**,** 207-211.

Navarrete-Mier,F., Sanz-Lazaro,C. & Marin,A. (2010) Does bivalve mollusc polyculture reduce marine fin fish farming environmental impact? *Aquaculture*, 306**,** 101-107.

Nicola,N.A. (1994) *Guidebook to citokynes and their receptors*. Oxfod.

Nordgarden,U., Fjelldal,P.G., Hansen,T., Bjornsson,B.T. & Wargelius,A. (2006) Growth hormone and insulin-like growth factor-I act together and independently when regulating growth in vertebral and muscle tissue of atlantic salmon postsmolts. *General and Comparative Endocrinology*, 149**,** 253-260.

Patruno,M., Maccatrozzo,L., Funkenstein,B. & Radaelli,G. (2006) Cloning and expression of insulin-like growth factors I and II in the shi drum (Umbrina cirrosa). *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 144**,** 137-151.

Patruno,M., Sivieri,S., Poltronieri,C., Sacchetto,R., Maccatrozzo,L., Martinello,T., Funkenstein,B. & Radaelli,G. (2008) Real-time polymerase chain reaction, in situ hybridization and immunohistochemical localization of insulin-like growth factor-I and myostatin during development of Dicentrarchus labrax (Pisces : Osteichthyes). *Cell and Tissue Research*, 331**,** 643-658.

Periago,M.J., Ayala,M.D., Lopez-Albors,O., Abdel,I., Martinez,C., Garcia-Alcazar,A., Ros,G. & Gil,F. (2005) Muscle cellularity and flesh quality of wild and fanned sea bass, Dicentrarchus labrax L. *Aquaculture*, 249**,** 175-188.

Peterson,B., Waldbieser,G. & Bilodeau,L. (2004) Muscle and liver IGF-I mRNA expression and plasma IGF-I levels in channel catfish administered rbGH over time. *Journal of Animal Science*, 82**,** 194.

Picchietti,S., Fausto,A.M., Randelli,E., Carnevali,O., Taddei,A.R., Buonocore,F., Scapigliati,G. & Abelli,L. (2009) Early treatment with Lactobacillus delbrueckii strain induces an increase in intestinal T-cells and granulocytes and modulates immune-related genes of larval Dicentrarchus labrax (L.). *Fish & Shellfish Immunology*, 26**,** 368-376.

Pierce,A.L., Beckman,B.R., Schearer,K.D., Larsen,D.A. & Dickhoff,W.W. (2001) Effects of ration on somatotropic hormones and growth in coho salmon. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 128**,** 255-264.

Pinnegar,J.K. & Polunin,N.V.C. (1999) Differential fractionation of delta C-13 and delta N-15 among fish tissues: implications for the study of trophic interactions. *Functional Ecology*, 13**,** 225-231.

Pinnegar,J.K. & Polunin,N.V.C. (2000) Contributions of stable-isotope data to elucidating food webs of Mediterranean rocky littoral fishes. *Oecologia*, 122**,** 399-409.

Rebhan,Y. & Funkenstein,B. (2008) Inhibition of fish myostatin activity by recombinant fish follistatin and myostatin prodomain: Potential implications for enhancing muscle growth in farmed fish. *Aquaculture*, 284**,** 231-238.

Reinecke,M. & Collet,C. (1998) The phylogeny of the insulin-like growth factors. *International Review of Cytology - A Survey of Cell Biology, Vol 183*, 183**,** 1-94.

Rollo,A., Sulpizio,R., Nardi,M., Silvi,S., Orpianesi,C., Caggiano,M., Cresci,A. & Carnevali,O. (2006) Live microbial feed supplement in aquaculture for improvement of stress tolerance. *Fish Physiology and Biochemistry*, 32**,** 167-177.

Saban,M.R., Memet,S., Jackson,D.G., Ash,J., Roig,A.A., Israel,A. & Saban,R. (2004) Visualization of lymphatic vessels through NF-kappa B activity. *Blood*, 104**,** 3228-3230.

Sanchez-Jerez,P., Bayle-Sempere,J., Fernandez-Jover,D., Valle,C. & Dempster,T. (2007) Ecological relationship between wild fish populations and Mediterranean aquaculture in floating cages. *Impact of mariculture on coastal ecosystems, CIESM Workshop Monographs No. 32* (ed F. Briand), Lisboa, 21-24 Feb.

Sanz-Lazaro,C. & Marin,A. (2006) Benthic recovery during open sea fish farming abatement in Western Mediterranean, Spain. *Marine Environmental Research*, 62**,** 374-387.

Schoeninger,M.J. & Deniro,M.J. (1984) Nitrogen and Carbon Isotopic Composition of Bone-Collagen from Marine and Terrestrial Animals. *Geochimica et Cosmochimica Acta*, 48**,** 625-639.

Smith,T.R., Tremblay,G.C. & Bradley,T.M. (1999) Hsp70 and a 54 kDa protein (Osp54) are induced in salmon (Salmo salar) in response to hyperosmotic stress. *Journal of Experimental Zoology*, 284**,** 286-298.

Triantafyllidis,A. (2007) Aquaculture escapes: new DNA based monitoring analyses and application on sea bass and sea bream. *Impact of mariculture on coastal ecosystems, CIESM Workshop Monographs No. 32* (ed F. Briand), Lisboa, 21-24 Feb.

Vianello,S., Brazzoduro,L., Dalla Valle,L., Belvedere,P. & Colombo,L. (2003) Myostatin expression during development and chronic stress in zebrafish (Danio rerio). *Journal of Endocrinology*, 176**,** 47-59.

Yang,L.H., Zha,J.M., Zhang,X.Y., Li,W., Li,Z.L. & Wang,Z.J. (2010) Alterations in mRNA expression of steroid receptors and heat shock proteins in the liver of rare minnow (Grobiocypris rarus) exposed to atrazine and p,p '-DDE. *Aquatic Toxicology*, 98**,** 381-387.

Yengkokpam,S., Pal,A.K., Sahu,N.P., Jain,K.K., Dalvi,R., Misra,S. & Debnath,D. (2008) Metabolic modulation in Labeo rohita fingerlings during starvation: Hsp70 expression and oxygen consumption. *Aquaculture*, 285**,** 234-237.

CONCLUSIONS

1. There is no effect on the growth of bivalves (*O. edulis* and *M. galloprovincialis*) when subjected to the influence of fish farming. Therefore, under the open water conditions studied the integration of bivalve cultures in fish farms is not a useful strategy to diminish fish farm wastes or their associated environmental impact.

2. Biofilm community is sensitive to fish farm influence, and undergoes structural and trophic changes in response. Biofilm structure and trophic diversity is influenced by seasonality as well as by the loading of fish farm wastes. In consequence, the biofilm community constitutes a reliable tool to assess the dissolved aquaculture waste impact.

3. The leaks of organic matter from the fish farms in floating net-cages alter the fish abundance and the fish trophic behavior by collapsing the niche width of the wild fish associated to this aquaculture activity.

4. In European sea bass specimens associated to fish farms, either the trophic behavior and expression profile of physiological-status-related genes differ from that in wild and cultured populations. This integrative approach constitutes a useful tool to assess the aquaculture impact on the welfare of fish populations.

ANEXOS

Aquaculture 306 (2010) 101–107

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Does bivalve mollusc polyculture reduce marine fin fish farming environmental impact?

Francisco Navarrete-Mier *, Carlos Sanz-Lázaro, Arnaldo Marín

Universidad de Murcia, Departamento de Ecología e Hidrología, 30100 Murcia, Spain

ARTICLE INFO

Article history:
Received 1 December 2009
Received in revised form 9 June 2010
Accepted 10 June 2010

Keywords:
Polyculture
Bivalve
Net cages
Stable isotopes
Metals
Mediterranean

ABSTRACT

The ability of bivalve culture in the proximity of an open water fin fish farm to reduce the environmental impact caused by organic wastes was tested. The experiment involved floating net cages containing cultured gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) in the western Mediterranean. Two bivalve species, oyster (*Ostrea edulis*) and mussel (*Mytilus galloprovincialis*), were deployed for 3 months along a distance transect running from 0 to 1800 m from the fish cages. Shell growth, flesh dry weight, the concentration of stable isotopes of carbon and nitrogen, and metal accumulation (Cd, Pb, Cu and Zn) in the bivalves were analyzed. Bivalves showed significant growth compared with their respective starting sizes, although closeness to the fish farm did not enhance such growth. The stable isotopes content indicated that there was no relationship between the main input of organic matter from the fish farm (the feed) and the trophic behavior of the bivalves. Neither did metal accumulation show a trend along the distance gradient from the fish farm. All the results were consistent in indicating that neither oysters nor mussels fed on fin fish farming wastes. This work demonstrates that the polyculture of fin fish and bivalves does not represent an appropriate tool for reducing the environmental impact of fin fish aquaculture in open water.

1. Introduction

Intensive open water fish culture is a common method of aquaculture in Europe (Mantzavrakos et al., 2007), particularly in the Mediterranean Sea (Vita et al., 2004), where this activity has sharply increased since 1990 and is expected to rise by 5% annually in the next two decades (FAO, 2006).

Aquaculture has a negative environmental impact due to the release of great amounts of particulate organic matter in the form of suspended detritus (Karakassis et al., 2000; Mazzola and Sarà, 2001), which mainly consists of uneaten feed and excretion products from the cultured fish (Cheshuk et al., 2003; Hall et al., 1992; Holby and Hall, 1991). Most of these wastes are accumulated on the seabed close to the fish farms, causing severe modifications of the physical and chemical characteristics of the benthic environment (Diaz, 2001; Karakassis et al., 2000; Rosenberg et al, 2001; Sanz-Lázaro and Marín, 2008). The result is usually a reduction in the number of species and biodiversity, accompanied by changes in the trophic structure (Otaway, 1995; Sanz-Lázaro and Marín, 2006) and algal blooms in the water column (Angel et al., 2002).

Thus, one of the major challenges for the sustainable development of aquaculture is the minimization of its environmental impact. Several authors have proposed alternatives such as polyculture systems (Jones and Iwama, 1991; Mazzola and Sarà, 2001; Neori et al., 2000). The value of polyculture has been emphasized as an ecological engineering practice to restrain the environmental impact of waste from fish cultivation by recycling particulate and dissolved matter, and to enhance the total farming productivity (Troell et al., 2003).

Organic carbon in the form of uneaten feed and faecal material produced by cultured fish may represent a source of available food for filter-feeding organisms, such as bivalves. Kautsky et al. (1997) proposed an open water integrated culture system whereby filter-feeding bivalves are cultured adjacent to fish floating cages. This system was expected to reduce nutrient loadings by filtering and assimilating particulate wastes (fish feed and faeces) as well as phytoplankton. Some of the waste nutrients released to the local environment would be removed when the cultured bivalves are harvested. In this way, the bivalves would perform as biological filters and environmental cleaners (Neori et al., 1998, 2000; Shpigel and Blaylock, 1991; Shpigel and Neori, 1996). Thus, the authors concluded that integrated aquaculture could, on the one hand, increase the productivity of bivalve culture and, on the other, reduce fish farm waste loadings and therefore the environmental impact of the same. In the Mediterranean, intensive fish farming could, for example, be accompanied by the culture of native species of bivalves such as, *Ostrea edulis* and *Mytilus galloprovincialis*.

The concept of an integrated bivalve-fish culture model is attractive and appears to offer promising prospects. A few studies

* Corresponding author. Tel.: +34 868884977; fax: +34 868883639.
E-mail address: kfrancisco.navarrete@um.es (F. Navarrete-Mier).

0044-8486/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2010.06.013

regarding the potential of polyculture in open water have been undertaken, although the conclusions are not unequivocal. Some authors suggest that bivalves could use fish farm wastes as an additional food supply (Mazzola and Sarà, 2001; Peharda et al., 2007), resulting, for example in the increased growth of mussels (Wallace, 1980) and oysters (Jones and Iwama, 1991) cultured adjacent to fish cages. On the other hand, some authors found no significant relationship between the floating fish cages and bivalve growth or bioaccumulation in bivalves (Cheshuk et al., 2003; Stirling and Okumus, 1995). Hence, the question whether integrated bivalve-fish culture reduces the environmental impact of open water aquaculture remains unresolved.

In recent years, stable isotope analyses have emerged as reliable tools for elucidating trophic structures and inferring pathways of energy flow in food webs (Cifuentes et al., 1988; Fry, 2006). The origin of organic matter deposits in coastal environments has often been elucidated using measurements of $\delta^{13}C$ (carbon) and $\delta^{15}N$ (nitrogen) as stable isotopic composition markers (Fry et al., 1977; Kennedy et al., 2004; Papadimitriou et al., 2005; Sweeney and Kaplan, 1980; Thornton and McManus, 1994). Therefore, the measurement of stable isotopes as markers is one of the most effective methods for ascertaining carbon sources and trophic relationships among organisms (Lojen et al., 2005; Schwarcz and Schoeninger, 1991; Yoshii et al., 1999), and, in our case, for the analysis of the extent to which fish farm organic wastes are used by bivalves. This method involves a comparison of stable isotope ratios between consumers and the food supply, and depends on differences in isotopic values between the different food sources (Deegan and Garritt, 1997). Stable isotope analysis has been used with persistent chemicals, such as metals, to relate chemical contamination with trophic community structure (Marín-Guirao et al., 2008). Bivalves are well known bioaccumulators of metals (Daskalakis, 1996; Shulkin et al., 2003), a fact that can be used as a complementary tool for assessing fish farm effects, since the diet of cultured fish usually contains a range of trace metals (Dean et al., 2007) and aquaculture also uses compounds containing metals in their activities (net antifouling, paints, fuels, etc). Analysis of elements such as Cu, Cd, Zn and Pb in bivalve tissues would confirm the assimilation of products from fish farming (Watanabe et al., 2008).

The aim of this study was to evaluate the usefulness of the polyculture, involving the bivalves *M. galloprovincialis and O. edulis* in an open water fish farm raising *Dicentrarchus labrax* and *Sparus aurata*, to reduce the environmental impact of the fish farm. To do this, we analyzed different bivalve parameters along an environmental gradient: i) growth ii) stable isotope concentrations and iii) the bioaccumulation of metals (Cu, Cd, Zn, and Pb) due to fish farming.

2. Materials and methods

2.1. Study area

The study was conducted in the surroundings of a marine fish farm located in Águilas, SE Spain, (western Mediterranean; 37° 24′ 56.2″ N, 1° 32′ 4.0″ W) (Fig. 1), with an average annual production of 1000 tonnes. The farm produces gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) in a 12 net cage system, each cage measuring 25 m in diameter and 19 m in depth. The water temperature at 6 m depth ranges between 21 and 26 °C and the salinity from 37 to 38. The seabed consists of carbonate sand with an unattached coralline algae community, at a mean depth of 31 m.

2.2. Experimental design

The study was carried out with flat oyster (*Ostrea edulis*, Linnaeus, 1758) and Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) obtained from a commercial shellfish farm located in a non-polluted area close to a marine protected area in Santa Pola (Alicante, Spain).

The bivalves were individually measured and placed in commercial cylindrical culture cages containing trays of 0.40 m diameter and 0.12 m high. Bivalves were tagged and placed individually in eight compartments per cage. All specimens were healthy juveniles with initial shell lengths of 479 ± 58 and 444 ± 47 mm (mean $\pm$ SD) for oysters and mussels, respectively. The biometrics measurements were also registered at the end of the experimental time following the methodology described by Helm et al. (2004) and Hwang et al. (2007). A total of 112 individuals per bivalve species were measured individually.

The bivalves were deployed in the field during three months (from March 13 to June 11, 2008). The bivalves were placed 0, 25, 120, 300, 600 and 1800 m from the net cages to assess the influence of the fish farm on their development (Fig. 1). The bivalve cages were maintained at 15 m depth to ensure that the animals were not influenced

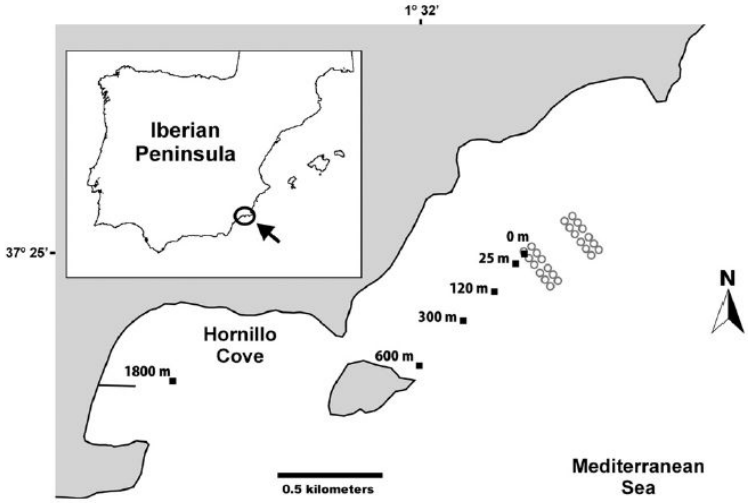

Fig. 1. Location of the fish farm and the sampling stations where the bivalves were deployed.

by re-suspension from the seabed. In the laboratory, the bivalves were individually processed for biometric analysis, which focused on growth, measured as length, and dry weight (Helm et al., 2004; Hwang et al., 2007). The bivalves were dissected to separate valves and eliminate the digestive tract. The remaining tissues were rinsed with distilled water, dried by lyophilization, grounded to a powder and stored at −20 °C until analysis (Riera and Richard, 1996). Samples of the fish feed used in the fish farm during the study period were collected and handled in the same way.

2.3. Spread and analysis of particulate wastes

The dispersion of fish farm particulate wastes was studied to ensure that the bivalves were located at distances from the fish farm that were representative of an environmental gradient due to fish farming. To do this, the currents and the sedimentation rates were measured. The currents were measured by means of a current meter (Valeport 106 current meter, Valeport Limited, Dartmouth, UK; located within in the limits of the fish farm next to the fish cages at 15 m depth) during the whole experiment. The sedimentation rates of particulate organic carbon (POC) were measured to analyze the reach and extent of particulate waste dispersion. POC sedimentation rates were obtained by means of sedimentation traps placed along the same spatial gradient as the bivalves at increasing distances from the fish farm (0, 20, 120 and 600 m). The sedimentation traps were made up of four attached cylinders (100 cm height and 12 cm diameter). Each cylinder had a funnel at the bottom, which guided the particulate matter into a 250 ml polyethylene tube, which was too narrow to allow the passage of fish. By means of a mooring system, the sedimentation traps were installed 3 m above the seabed to avoid resuspension (Fig. 2). Five to seven samples, were taken every 5 days, at each station.

2.4. POC analyses

The particles collected from the sedimentation traps were dried in an oven (60 °C) to constant weight before and were then finely ground. After a pre-treatment consisting of adding 1:1 HCl, POC was determined using a Carlo Erba Inst. EA 1108 Elemental Analyser (Carlo Erba Strumentazione, Milan, Italy).

2.5. Stable isotope ratio analyses

The carbon and nitrogen isotope ratios of the samples were measured with a Flash EA1112 (ThermoFinnigan) elemental analyzer connected to a Delta^{plus} mass spectrometer of isotopic relationships (ThermoFinnigan).

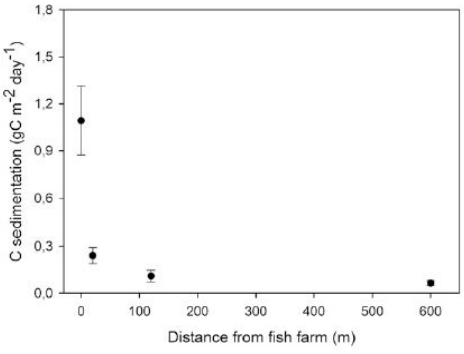

Fig. 2. Carbon sedimentation rates (mean ± SE).

All the isotopic data are reported in the conventional δ notation as follows:

$$\delta^{13}C \text{ or } \delta^{15}N = \left(R_{sample} / R_{standar} - 1\right) 1000\ (‰)$$

where R represents the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio for $\delta^{13}C$ and $\delta^{15}N$, respectively. All $\delta^{13}C$ values were reported as the deviation relative to the Vienna Pee Dee Belemnite Limestone Standart (v-PDB). The $\delta^{15}N$ standards were calibrated and results are reported relative to atmospheric nitrogen.

2.6. Metals analyses

For metal analysis, 0.2 g each of freeze dried mussel flesh, oyster flesh or feed was weighed and placed in a Teflon reactor. After the addition of 3 ml H_2O Milli-Q water, 5 ml concentrated nitric acid (Merk Suprapur), and 2 ml H_2O_2 at 30%; the reactor was maintained in a microwave digester for 20 min with a maximum temperature of 210 °C.

Once the sample was cold, the volume was adjusted to 25 ml with Milli-Q water and transferred to a low density polyethylene container. It was then stored at 4 °C until analysis in a spectrophotometer.

Cu, Cd, Pb and Zn were determined by an inductively coupled plasma-mass spectrometer (ICP-MS Algilent 7500 ce, with Octopole reaction system). The detection limits were: Cu, 0.7 μg g^{-1}; Zn, 1.8 μg g^{-1}; Cd, 0.04 μg g^{-1}; Pb, 0.05 μg g^{-1}. The metal concentrations in soft tissues of the bivalves are reported in μg g^{-1} of dry weight (Maanan, 2008).

2.7. Statistical analyses

After checking for normality with the Kolmogorov–Smirnov test and homogeneity of variance with the Levene test, one-way analysis of variance (ANOVA) was performed to identify significant differences between stations with regards to growth, stable isotope and metal accumulation in the organisms studied. When significant differences were found between stations, the Bonferroni's test *post hoc* test was performed. To analyse differences between initial and final concentration of metals a one way ANOVA also was used, followed by a Bonferroni's test post hoc test. In this case comparisons were only performed between the initial value and the value recorded in each sampling station individually after 90 days. All statistical tests were performed with a significance level of $\alpha = 0.05$.

3. Results

3.1. Spread and analysis of particulate waste analyses

The data of the currents showed that the main current was parallel to the coast in both directions (NE and SW). The mean and maximum current speeds along the distance gradient, where the bivalves were deployed (SW direction), were 0.07 and 0.85 m s^{-1}, respectively. So the data concerning the currents confirmed that the cages containing the bivalves were placed along the main current and were thus influenced by the fish farm wastes.

The sedimentation rate of POC was 1.09, 0.24, 0.11 and 0.06 g m^{-2} day^{-1} at 0, 20, 120 and 600 m from the net cages, respectively, following the same distance gradient as that for the studied bivalves (Fig. 2). POC sedimentation rates were notably higher below the fish farm than in the rest of the sampling points, where they decreased with distance from the fish farm, following an exponential decay. The behaviour of the POC sedimentation rates along gradient from the fish farm was comparable to that observed in other fish farms (Holmer et al., 2007). These results indicate

that the distance gradient where the bivalves were placed for this study was representative of an environmental gradient due to fish farming.

3.2. Biometric analyses

The oysters and mussels showed a significant increase in the shell length of 85 ± 54 mm and 152 ± 45 mm, respectively, between the beginning and the end of the experiment (90 days). The flesh dry weight also decreased by 0.13 ± 0.07 g for oysters and 0.13 ± 0.04 g for mussels. Neither oysters nor mussels did showed any significant differences in shell length or flesh dry weight (Figs. 3 and 4) along the gradient.

3.3. Metal analyses

The initial metal concentrations in the bivalves were analyzed before the field assays (oysters: Cd = 1.70 ± 0.29 μg g^{-1}; Cu = 112.06 ± 22.25 μg g^{-1}; Pb = 0.91 ± 0.15 μg g^{-1}; Zn = 681.03 ± 150.67 μg g^{-1}; mussels: Cd = 0.35 ± 0.07 μg g^{-1}; Cu = 4.72 ± 0.73 μg g^{-1}; Pb = 1.16 ± 0.16 μg g^{-1}; Zn = 83.92 ± 16.59 μg g^{-1}). The metal concentrations determined in the feed were Cd = 0.24 ± 0.02 μg g^{-1}; Cu = 8.48 ± 0.33 μg g^{-1}; Pb = 0.15 ± 0.02 μg g^{-1} Zn = 83.02 ± 0.77 μg g^{-1}. For oysters there were no significant differences between the initial and the final metal concentrations at any of the sites. The same was true for mussels, with the exception of the Pb whose initial and final concentrations showed significant differences in most of the sites (Bonferroni's test, $p<0.05$). Oysters accumulated Cu, Zn and Cd to a greater extent than mussels, but Pb to a lower extent. Metal accumulation was compared between sites, the data pointed to no pattern related with distance from the fish farm and no significant differences between the sites for either oysters or mussels (Fig. 5).

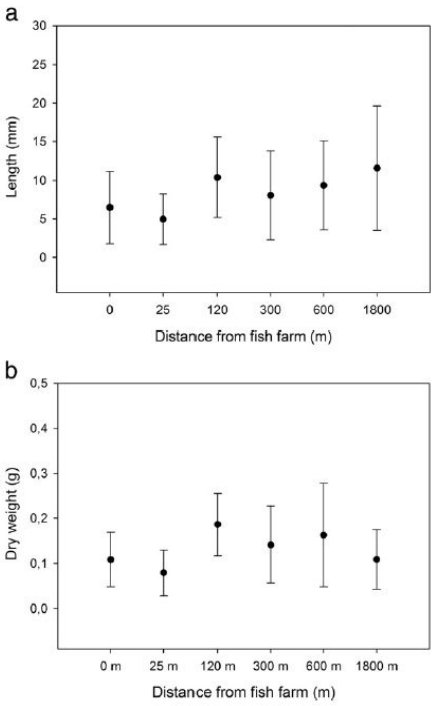

Fig. 3. Oyster growth along the distance gradient from the fish farm (mean ± SE, n = 12). Growth is shown as the increase in a) length of shell and b) mean dry weight, after 90 days.

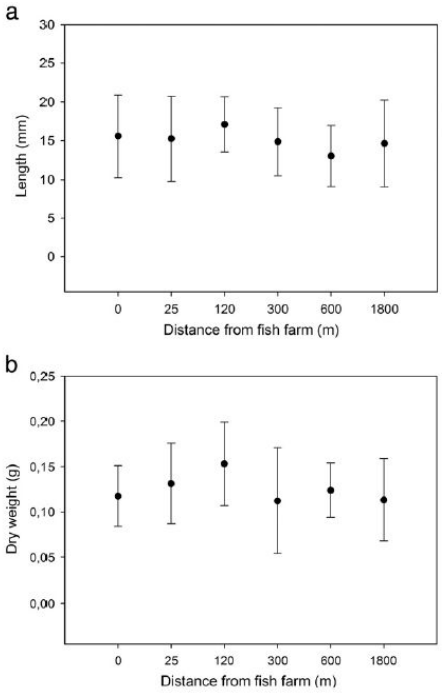

Fig. 4. Mussel growth along the distance gradient from the fish farm (mean ± SE, n = 12). Growth is shown as the increase in a) length of shell and b) mean dry weight, after 90 days.

3.4. Stable isotopic analyses

The stable isotope composition of the bivalves did not differ significantly between stations. The nitrogen and carbon ratios for oysters at all sites appears in a narrow range ($\delta^{13}C = 2.7‰$; $\delta^{15}N = 0.9‰$) (Fig. 6), while for mussels the range was much narrowed ($\delta^{13}C = 1.8‰$; $\delta^{15}N = 1.3‰$) (Fig. 7). The $\delta^{13}C$ and $\delta^{15}N$ values for oysters and mussels were very different from the values of the feed, which had a lower $\delta^{13}C$ (−22.3 ± 0.5‰) and a higher $\delta^{15}N$ (5.3 ± 0.4‰). Moreover, the isotopic composition of both bivalve species was not influenced by the fish farm wastes.

4. Discussion

The present study found no significant influence of fish farm wastes on the growth of *O. edulis* and *M. galloprovincialis*. After three months, the oysters and mussels showed significant growth compared with measurements made at the beginning of the experiment, which indicates good adaptation and an active metabolism (Hwang et al., 2007; Wallace, 1980) (Figs. 3 and 4). Bivalve growth was similar for all the stations along the field transect, implying that food availability was not greater due to fish farm wastes. Several authors have mentioned that fish farms increase the organic matter in the ecosystem, in general. This, in turn results in an increase in the food

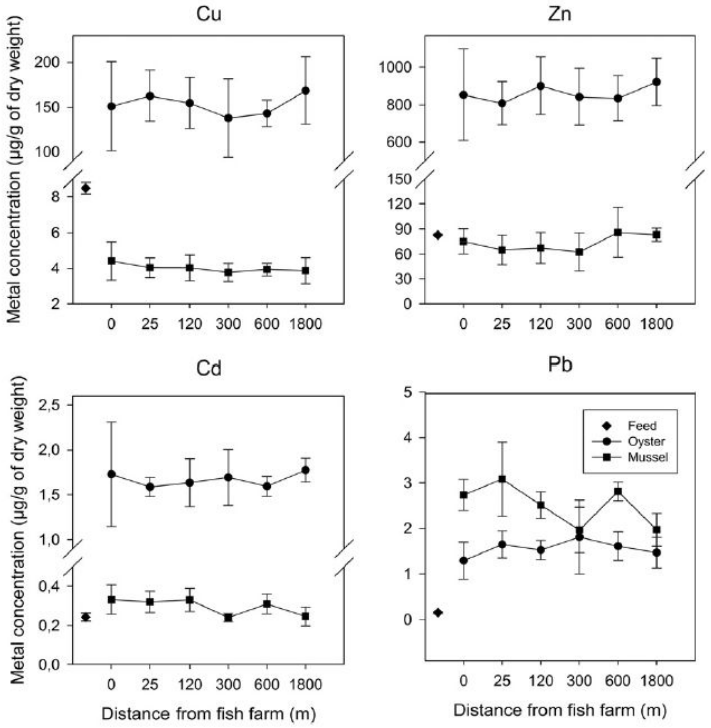

Fig. 5. Cu, Zn, Cd and Pb concentrations in oysters, mussels and fish feed after 90 days (mean ± SE, n = 4 for bivalves, n = 3 for feed).

availability for filter-feeding organisms and, consequently, an increase in the growth rate of these organism (Cheshuk et al., 2003; Jones and Iwama, 1991; Mazzola and Sarà, 2001; Neori et al., 1998, 2000). Taylor et al. (1992) in *M. edulis* and Cheshuk et al. (2003) in *M. planulatus*, both observed that the distance to fish cages dedicated to *Salmo salar* culture did not influence the growth of these bivalves. Conversely, Jones and Iwama (1991) observed increased growth in *Crassostrea gigas* near a culture of *Oncorhynchus tshawytscha*. Also, Peharda et al. (2007) found increased growth in *M. galloprovincialis* in a farm of *D. labrax* and *S. aurata*.

As in the case of growth the stable isotope composition of the bivalves did not differ significantly between sites. Stable isotopes are widely used as time-integrating tracers of trophic interactions, as well as to monitor relationships in coastal ecosystems (Lefebvre et al., 2009). Thus, the isotopic analyses showed that bivalves did not change their diet because of the closeness to the farm. Oysters and mussels both had a similar level of $\delta^{13}C$ and $\delta^{15}N$, indicating that the carbon sources and the trophic niche were stable along the transect from the fish farm. Furthermore, isotope levels also indicated that there was no relationship between the main input of organic matter from the fish farm (fish feed) and the trophic behaviour of the bivalves. These results concur with the view that the excess of organic matter exported by the fish farm is not used by the bivalves close the facility. Rather, such enrichment is more likely to make a significant

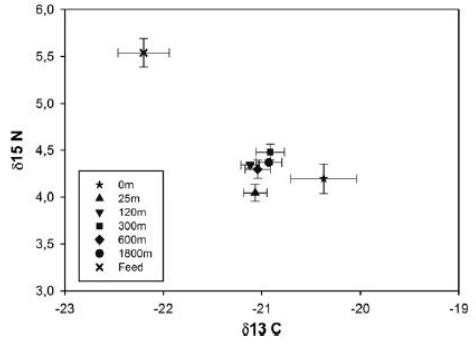

Fig. 6. Bi-plot of $\delta^{13}C$ and $\delta^{15}N$ (mean ± SE, n = 4 for bivalves, n = 3 for feed) of oysters and fish feed after 90 days.

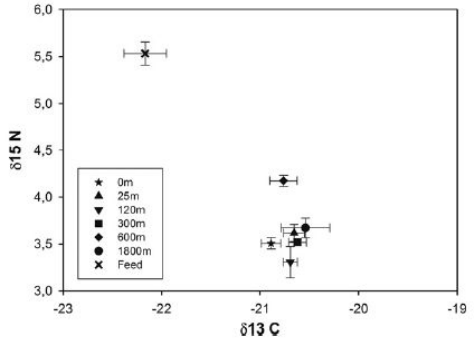

Fig. 7. Bi-plot of $\delta^{13}C$ and $\delta^{15}N$ (mean ± SE, n = 4 for bivalves, n = 3 for feed) of mussels and fish feed after 90 days.

contribution to the production of phytoplankton several kilometres away down current (Danovaro et al., 2004; Holmer et al., 2007).

The assessment of metal accumulation confirmed this view. It has been observed that bivalves in general tend to accumulate metals (Daskalakis, 1996; Shulkin et al., 2003). In sediments around cages, Dean et al. (2007) found that the gradient of metal concentration increased with proximity to the fish farm. Thus, if the organisms used in our experiment were affected by the farm, they should have showed similar behaviour. However, there were no significant differences in the metal concentration of metals, which clearly suggests a different supply of resources for the tested animals. No differences in metal accumulation was found between initial and final values in oysters, and the same being true for mussels with the exception of Pb in most of the stations. These data could imply a greater availability of this metal in the studied area. We found no differences in Pb between the mussels from all the stations, which suggest that Pb enrichment was not due to the impact of the fish farm.

Our results suggest that systems involving the polyculture of bivalves and fish need close analysis before they are implemented. Such polyculture may not only be irrelevant for diminishing the environmental impact derived from fish culture, but they might even generate other impacts, such as a higher load of organic matter from the pseudo-faeces of bivalves, an increased risk of disease to fish and deterioration of the cultivation gear through fouling (Cranford et al., 2009; Hatcher et al., 1994; Mirto et al., 2000). Bivalve integrated coastal aquaculture systems could relieve the eutrophication of coastal water if they are harvested and brought ashore. Although mussels and oysters do not seem to directly incorporate fish farm wastes, the nutrient budget of a water body could benefit from extractive aquaculture (Lindahl et al., 2005) because they feed on phytoplankton, which take up nutrients from the water. However, this profit may only be effective in enclosed water bodies with high nutrient inputs and low seawater exchange.

In conclusion, our study strongly suggests that there is no effect on the growth of bivalves (*O. edulis* and *M. galloprovincialis*, in this case) when subjected to the influence of fish farming. The C and N stable isotope composition and metal concentration demonstrated that these bivalves did not assimilate organic wastes from the studied fish farm, which suggests that the positive effects observed in other geographical areas with limited hydrodynamism were probably due to indirect causes (*e.g.* plankton bloom). Therefore, under open water conditions the integration of bivalves culture in fish farms does not seem to be a useful strategy for diminishing fish farm wastes or for lessening any associated environmental impact.

Acknowledgements

The authors are grateful to the management of Culmarex S.A. who provided farm access and assistance during the field phase of this study. We would also like to thank P. Castillo-Briceño, J. Lloret, M.D. Belando, C. Gutierrez-Cánovas and D. Celdrán for their useful comments. The work was supported by the Programme AlBan, the European Union Programme of High Level Scholarships for Latin America (FNM scholarship No. E07D401236EC) and by FPU predoctoral grant of the Ministerio de Ciencia y Tecnología from Spain.

References

Angel, D.L., Aden, N., Breitstein, S., Yerman, A., Katz, T., Spanier, E., 2002. In situ biofiltration: a means to limit the dispersal of effluents from marine finfish cage aquaculture. Hydrobiologia 469, 1–10.

Cheshuk, B.W., Purser, G.J., Quintana, R., 2003. Integrated open-water mussel (*Mytilus planulatus*) and Atlantic salmon (*Salmo salar*) culture in Tasmania, Australia. Aquaculture 218, 357–378.

Cifuentes, L.A., Sharp, J.H., Fogel, M.L., 1988. Stable carbon and nitrogen isotope biogeochemistry in the Delaware estuary. Limnology and Oceanography 33 (5), 1102–1115.

Cranford, P.K., Hargrave, B.T., Doucette, L.I., 2009. Benthic organic enrichment from suspended mussel (*Mytilus edulis*) culture in Prince Edward Island, Canada. Aquaculture 292, 189–196.

Danovaro, R., Gambi, C., Luna, G.M., Mirto, S., 2004. Sustainable impact of mussel farming in the Adriatic Sea (Mediterranean Sea): evidence from biochemical, microbial and meiofaunal indicators. Marine Pollution Bulletin 49, 325–333.

Daskalakis, K.D., 1996. Variability of metal concentrations in oyster tissue and implications to biomonitoring. Marine Pollution Bulletin 32, 794–801.

Dean, R.J., Shimmield, T.M., Black, K.D., 2007. Copper, zinc and cadmium in marine cage fish farm sediments: an extensive survey. Environmental Pollution 145, 84–95.

Deegan, L.A., Garritt, R.H., 1997. Evidence for spatial variability in estuarine food webs. Marine Ecology Progress Series 147, 31–47.

Diaz, R.J., 2001. Overview of hypoxia around the world. Journal of Environmental Quality 30, 275–281.

FAO, 2006. The state of World Fisheries and Aquaculture 2006. Rome: United Nations. Food and Agricultural Organization.

Fry, B., 2006. Stable isotope ecology. Springer, New York, New York, USA.

Fry, R., Scalan, R.S., Parker, P.L., 1977. Stable carbon isotope evidence for two sources of organic matter in coastal sediments: seagrasses and plankton. Geochimica el Cosmochimica Acta 41, 1875–1877.

Hall, P.O.J., Holby, O., Kollberg, S., Samuelsson, M.O., 1992. Chemical fluxes and mass balances in a marine fish cage farm. IV. Nitrogen. Marine Ecology Progress Series 89, 81–91.

Hatcher, A., Grant, J., Schofield, B., 1994. Effects of suspended mussel culture (*Mytilus* spp.) on sedimentation, benthic respiration and sediment nutrient dynamics in a coastal bay. Marine Ecology Progress Series 115, 219–235.

Helm, M., Bourne, N., Lovatelli, A., 2004. Hatchery culture of bivalves. A practical manual. Food and Agriculture Organization of the United Nations, Roma.

Holby, O., Hall, P.O.J., 1991. Chemical fluxes and mass balances in a marine fish cage farm. II. Phosphorus. Marine Ecology Progress Series 70, 263–272.

Holmer, M., Marba, N., Diaz-Almela, E., Duarte, C.M., Tsapakis, M., Danovaro, R., 2007. Sedimentation of organic matter from fish farms in oligotrophic Mediterranean assessed trough bulk and stable isotope ($\delta^{13}C$ and $\delta^{15}N$) analyses. Aquaculture 262, 268–280.

Hwang, J.J., Yamakawa, T., Aoki, I., 2007. Growth of wild pearl oysters *Pinctata fucata*, *Pinctata margaritifera* and *Pinctata sugillata* (Bivalvia: Pteriidae) in Taiwan. Fisheries Science 73, 132–141.

Jones, T.O., Iwama, G.K., 1991. Polyculture of the Pacific oyster, *Crassostrea gigas* (Thunberg), with chinook salmon *Oncorhynchus tshawytscha*. Aquaculture 92, 313–322.

Karakassis, I., Tsapakis, M., Hatziyanni, E., Papadopoulou, K.-N., Plaiti, W., 2000. Impact of cage farming of fish on the seabed in three Mediterranean coastal areas. ICES Journal of Marine Science 57, 1462–1471.

Kautsky, N., Troell, M., Folke, C., 1997. Ecological engineering for increased production and environmental improvement in open sea aquaculture. In: Etnier, C., Guterstam, B. (Eds.), Ecological Engineering for Wastewater Treatment. Lewis Publisher, Chelsea, Michigan, p. 496. Chapter 36.

Kennedy, H., Gacia, E., Kennedy, D.P., Papadimitriou, S., Duarte, C.M., 2004. Organic carbon sources to SE Asian coastal sediments. Estuarine, Coastal and Shelf Science 60, 59–68.

Lefebvre, S., Harma, C., Blin, J.-L., 2009. Trophic typology of coastal ecosystems based on $\delta^{13}C$ and $\delta^{15}N$ ratios in an opportunistic suspension feeder. Marine Ecology Progress Series 390, 27–37.

Lindahl, O., Hart, R., Hernroth, B., Kolberg, S., Loo, L.-O., Olrog, L., Rehnstam-Holm, A.-S., Svensson, J., Svensson, S., Syversen, U., 2005. Improving marine water quality by mussel farming – a profitable solution for Swedish society. Ambio 34 (2), 129–136.

Lojen, S., Spanier, E., Tsemel, A., Katz, T., Eden, N., Angel, D.L., 2005. $\delta^{15}N$ as a natural tracer of particulate nitrogen effluents released from marine aquaculture. Marine Microbiology 148, 87–96.

Maanan, M., 2008. Heavy metal concentrations in marine molluscs from the Morocan coastal region. Environmental Pollution 153, 176–183.

Mantzavrakos, E., Kornaros, M., Lyberatos, G., Kaspis, P., 2007. Impacts of a marine fish faro in Argolikos Gula (Greece) on the water column and the sediment. Desalination 210, 110–124.

Marín-Guirao, L., Lloret, J., Marin, A., 2008. Carbon and nitrogen stable isotopes and metal concentration in food webs from a mining-impacted coastal lagoon. The Science of the Total Environment 393, 118–130.

Mazzola, A., Sarà, G., 2001. The effect of fish farming organic waste on food availability for bivalve molluscs (Gaeta Gulf, Central Tyrrhenian, MED): stable carbon isotopic analysis. Aquaculture 192, 361–379.

Mirto, S., La Rosa, T., Danovaro, R., Mazzola, A., 2000. microbial and meiofaunal response to intensive mussel-farm biodeposition in coastal sediments of the western Mediterranean. Marine Pollution Bulletin 40 (3), 244–252.

Neori, A., Ragg, N.L.L., Shipigel, M., 1998. The integrated culture of seaweed, abalone, fish and clams in modular intensive land-based systems: II. Performance and nitrogen partitioning within an abalone (*Haliotis tuberculata*) and macroalgae culture system. Aquacultural Engineering 17, 215–239.

Neori, A., Shpigel, M., Ben-Ezra, D., 2000. A sustainable integrated system for culture of fish, seaweed and abalone. Aquaculture 186, 279–291.

Otaway, N.M., 1995. Assessing impacts of deepwater sewage disposal: a cause study from New South Wales, Australia. Marine Pollution Bulletin 31, 347–354.

Papadimitriou, S., Kennedy, H., Kennedy, D.P., Duarte, C.M., Marbá, N., 2005. Sources of organic matter in seagrass-colonized sediments: a stable isotope study of the silt and clay fraction from *Posidonia oceanica* meadows in the western Mediterranean. Organic Geochemistry 36, 949–961.

Peharda, M., Zupan, I., Bavcevic, L., Frankic, A., Klanjscek, T., 2007. Growth and condition index of mussel *Mytilus galloprovincialis* in experimental integrated aquaculture. Aquaculture Research 38, 1714–1720.
Riera, P., Richard, P., 1996. Isotopic determination of food sources of *Crassostrea gigas* along a trophic gradient in the Estuarine Bay of Marennes-Oléron. Estuarine, Coastal and Shelf Science 42, 347–360.
Rosenberg, R., Nilsson, H.C., Diaz, R.J., 2001. Response of bentic fauna and changing sediment redox profiles over a hypoxic gradient. Estuarine, Coastal and Shelf Science 53, 343–350.
Sanz-Lázaro, C., Marín, A., 2006. Benthic recovery during open sea fish farming abatement in Western Mediterranean, Spain. Marine Environmental Research 62, 347–387.
Sanz-Lázaro, C., Marín, A., 2008. Assessment of finfish aquaculture impact on the benthic communities in the Mediterranean Sea. Dynamic Biochemistry, Process Biotechnology and Molecular Biology 2. Ed Global Science Books. (Special Issue I), pp. 21–32.
Schwarcz, H.P., Schoeninger, M., 1991. Stable isotope analyses in human nutritional ecology. Yearbook of Physical Antrhopology 34, 283–321.
Shpigel, M., Blaylock, R.A., 1991. The Pacific oyster, Crassostrea gigas, as a biological filter for a marine fish aquaculture pond. Aquaculture 92, 187–197.
Shpigel, M., Neori, A., 1996. The integrated culture of seaweed, abalone, fish and clams in modular intensive land-based systems: I. Proportions of size and projected revenues. Aquacultural Engineering 15 (5), 313–326.
Stirling, H.P., Okumus, I., 1995. Growth and production of mussels (*Mytilus edulis* L.) suspended at salmon cages and shellfish farms in two Scottish sea lochs. Aquaculture 134, 193–210.
Shulkin, V.M., Presley, B.J., Kavun, V.Ia., 2003. Metal concentrations in mussel *Crenomytilus grayanus* and oyster *Crassostrea gigas* in relation to contamination of ambient sediments. Environment International 29, 493–502.
Sweeney, R.E., Kaplan, I.R., 1980. Natural abundances of ^{15}N as a source indicator for near-shore marine sedimentary and dissolved nitrogen. Marine Chemistry 9, 81–94.
Taylor, B.E., Jamieson, G., Carefoot, T.H., 1992. Mussel culture in British Columbia: The influence of salmon farms on growth of *Mytilus edulis*. Aquaculture 8, 51–66.
Thornton, S.F., McManus, J., 1994. Application of organic carbon and nitrogen stable isotope and C/N ratios as source indicators of organic matter provenance in estuarine systems: evidence from the Tay Estuary, Scotland. Estuarine, Coastal and Shelf Science 38, 219–233.
Troell, M., Halling, C., Neori, A., Chopin, T., Bushcmann, A.H., Kaustky, N., Yarish, C., 2003. Integrated mariculture: asking the right questions. Aquaculture 226, 69–90.
Vita, R., Marín, A., Madrid, J.A., Jiménez-Brinquis, B., Cesar, A., Marín-Guirao, L., 2004. Effects of wild fishes on waste exportation from a Mediterranean fish farm. Marine Ecology Progress Series 277, 253–261.
Yoshii, K., Melnik, N.G., Timoshkin, O.A., Bondarenko, N.A., Anoshko, P.N., Yoshioka, T., Wada, E., 1999. stable isotope analyses of pelagic food web in Lake Baikal. Limnology and Oceanography 44, 502–511.
Wallace, J., 1980. Growth rates of different populations of the edible mussel, *Mytilus edulis*, in north Norway. Aquaculture 19, 303–311.
Watanabe, K., Monaghan, M.T., Takemon, Y., Omura, T., 2008. Biodilution of heavy metals in a stream macroinvertebrate food web: evidence from stable isotope analysis. Science of the Total Environment 394, 57–67.

Navarrete-Mier, Francisco. Marine Biology from the Catholic University of Ecuador, Sede Manabí (2002). MSc. Fish Aquaculture from the University of Murcia (2008). Currently he is taking a Postgraduate Programme of Fish Biology (Basic and Applied Aspects) in the University of Murcia, with a High Level scholarship from the AlBan Program, European Union. His work focuses on the study of alterations in secondary production as a result of the organic wastes generated by the gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) cultures, in floating net cages of SE Spain, using stable isotopes and gene expression as markers.

Sanz-Lázaro, Carlos. BSc Biology and MSc Marine Biology from the University of Alicante, Spain. Ph.D. from the University of Murcia (2009). Predoctoral grant (FPU, MEC). His research interests are focused on the impact of fish farms on the environment, which he studies from a holistic perspective, trying to characterize and integrate the components and environmental factors that play an important role in this process.

Marín, Arnaldo. PhD 1988 from the University of Murcia. Lecturer in Ecology at the University of Murcia since 1991. Vice Dean of Environmental Sciences. He has directed 14 doctoral theses. Author of 70 research articles.