Departamento de Ecología e Hidrología
Facultad de Biología
Universidad de Murcia

Consecuencias ecológicas del enriquecimiento por materia orgánica procedente de la acuicultura y de vertidos de petróleo en ecosistemas costeros

Tesis doctoral

Carlos Sanz Lázaro

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Ecological consequences of organic matter enrichment derived from aquaculture and oil spills in coastal ecosystems

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Memoria presentada para optar al grado de doctor en Biología por el licenciado

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D. José Francisco Calvo Sendín, Profesor Titular y Director del Departamento de Ecología e Hidrología,

INFORMA:

Que la Tesis Doctoral titulada "Consecuencias ecológicas del enriquecimiento por materia orgánica procedente de la acuicultura y de vertidos de petróleo en ecosistemas costeros", ha sido realizada por D. Carlos Sanz Lázaro, bajo la inmediata dirección y supervisión de D. Arnaldo Marín Atucha, 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 31 de marzo de 2009

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Fdo: José Fco. Calvo Sendín
Director del Departamento

Facultad de Biología
Departamento de Ecología e Hidrología
Campus Universitario de Espinardo. 30100 Murcia
T. 868 88 49 88 – F. 868 88 39 63 – www.um.es/ecologia

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D. Arnaldo Marín Atucha, Profesor Titular de Universidad del Área de Ecología en el Departamento de Ecología e Hidrología, AUTORIZA:

La presentación de la Tesis Doctoral titulada "Consecuencias ecológicas del enriquecimiento por materia orgánica procedente de la acuicultura y de vertidos de petróleo en ecosistemas costeros", realizada por D. Carlos Sanz Lázaro, bajo mi 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 31 de marzo de 2009

Fdo: Arnaldo A. Marín Atucha

Facultad de Biología
Departamento de Ecología e Hidrología

Campus Universitario de Espinardo. 30100 Murcia
T. 868 88 49 88 – F. 868 88 39 63 – www.um.es/ecologia

A mis padres

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RESUMEN GENERAL

El enriquecimiento de la materia orgánica, es una de las fuentes de contaminación más comunes, que puede ser generada por muy diversas actividades humanas. Las zonas costeras son zonas de alta productividad primaria (Viaroli et al., 1996; Underwood y Kromkamp, 1999; Chapman y Wang, 2001), consideradas como amenazadas y / o con regresión de los hábitats, ya que tienden a ser más gravemente afectadas por los contaminantes que otros ecosistemas porque los movimientos de las olas y las mareas pueden fomentar la acumulación de sustancias tóxicas. Los procesos biogeoquímicos que tienen lugar en los sedimentos costeros son relevantes, ya que están relacionados con los flujos de carbono y nutrientes a escala global. Toda la contaminación que afecte a estas zonas puede tener graves consecuencias en dichos ciclos.

El destino final de las partículas en suspensión es sedimentar y depositarse en el fondo del mar, donde se incorporan a la estratigrafía del sedimento. Aunque las fuentes de las partículas que componen el hábitat bentónico pueden variar espacial y temporalmente, con pocas excepciones, la fauna y las comunidades microbianas del bentos están estrechamente ligadas con la productividad de las aguas superficiales de los océanos (Solan y Wigham, 2005).

La entrada de las partículas desde la columna de agua al fondo del mar es baja y las comunidades bentónicas están mantenidas por tasas de entrada relativamente pequeñas de materia orgánica. Por lo tanto, cuando las tasas de entrada de materia orgánica aumentan, si la cantidad de materia orgánica es lo suficientemente grande, puede superar la capacidad de carga del ecosistema y de producir cambios sustanciales en los parámetros químicos de los sedimentos, así como en las comunidades bentónicas.

Dicha “sobrecarga” de materia orgánica, consume el oxígeno de la superficie del fondo marino, que produce hipoxia, y, si la tasa de deposición de carbono es demasiado alta, el fondo marino puede llegar a condiciones anóxicas. La consecuencia del consumo de oxígeno, es la aparición de bacterias con un metabolismo anaeróbico que utilizan, compuestos distintos al oxígeno como aceptores de electrones para obtener energía. Las bacterias predominantes de esos sedimentos orgánicamente enriquecidos, *Beggiatoa*, producen sulfuros como residuo de su metabolismo. Hay otras bacterias anaeróbicas menos abundantes que producen metano y amonio.

Todos estos subproductos, especialmente de sulfuros, son tóxicos en altas concentraciones para la fauna, y, junto con el agotamiento del oxígeno en el agua intersticial, pueden conducir al agotamiento de las especies más sensibles e incluso llegando a producirse incluso la desaparición de toda la fauna del sedimento (Brooks y Mahnken, 2003; Heilskov et al., 2006).

Las especies que habitan los sedimentos, especialmente la macrofauna, desempeñan un papel importante en el proceso de regeneración bentónica de nutrientes, que afectan a la producción primaria mediante el suministro de nutrientes y aumentan las tasas de reciclado pelágicas (Grall y Chauvaud, 2002). Algunas de estas especies producen bioturbación en los sedimentos. Básicamente, este proceso consiste en el movimiento y la mezcla de sedimentos, con lo que la redistribución de las partículas, como resultado de diferentes actividades, tales como el movimiento, la alimentación, construcción de tubos, etc. Los resultados de la bioturbación puede variar sustancialmente de estructuras muy simples a muy complejas, tales como galerías o tubos huecos de muchos tipos diferentes, dependiendo de las características bioturbadoras de las especies involucradas (Aller y Aller, 1998).

Las bacterias del sedimento, desempeñan un papel clave en los ciclos biogeoquímicos de los nutrientes. La bioturbación de la macrofauna puede influir en la diversidad, la estructura y función de estas bacterias (Findlay et al., 1990) y, a su vez, tener un efecto significativo sobre muchos de los procesos ecosistémicos (Biles et al., 2002). Esta actividad aumenta la superficie del sedimento expuesta al agua de la superficie, que es capaz de penetrar en capas más profundas de los fondos marinos, donde existe una baja actividad microbiana. Este proceso, produce un aumento de la oxidación del carbono de la materia orgánica anaeróbicamente refractaria (Kristensen, 2001), dando lugar a un incremento en la liberación de nutrientes, que apoyará la producción bento-pelágica primaria, aumentando así la producción bentónica (Solan y Wigham, 2005).

Dado que el potencial de la bioturbación de la fauna que habita el sedimento depende de la abundancia, biomasa y tipos particulares de bioturbación de cada especie, cualquier disminución de la macrofauna (parcial o total), puede disminuir la bioturbación y, por tanto, la irrigación mediada por la fauna, produciendo una disminución de la producción bentónica. Por encima de los niveles de entrada naturales de material particulado en un ecosistema bentónico, la cantidad de materia orgánica entrante extra, está positivamente correlacionada con el estrechamiento del estrato entre la superficie y la discontinuidad del potencial redox (RPD) (la profundidad desde la superficie del lecho marino hasta donde persiste un ambiente con oxígeno). Si la entrada de materia orgánica es suficientemente grande, todas las capas del sedimento hasta la superficie pueden quedarse en condiciones anóxicas (Pearson y Rosenberg, 1978).

Por lo tanto, debido al enriquecimiento orgánico, se producen importantes cambios en el sedimento, no sólo se producen cambios en los parámetros químicos (disminución de oxígeno y la estimulación del metabolismo anaeróbico de sedimentos) y biológicos (la sucesión hacia una comunidad con más especies oportunistas), sino también en relación con importantes funciones del ecosistema, tales como el reciclado de nutrientes y la mineralización de la materia orgánica.

Sin embargo, en algunos tipos de contaminación enriquecimiento orgánico, la materia orgánica está asociada con sustancias altamente tóxicas, como es el caso de los PAHs (hidrocarburos aromáticos policíclicos) en el petróleo. Debido a su alta toxicidad, estos compuestos generan cambios en los ecosistemas, incluso a menor cantidad de contaminantes. Sustancias como el petróleo, generan una toxicidad directa, y no sólo indirecta, derivada del aumento del consumo de oxígeno por parte del sedimento. Este hecho puede provocar respuestas diferentes en los ecosistemas afectados, en comparación con el enriquecimiento de materia orgánica "típico" que no contiene sustancias altamente tóxicas.

Los tests de toxicidad son ampliamente utilizados en la contaminación por petróleo, y los bioensayos usando crustáceos anfípodos se han convertido en un punto de referencia para la caracterización de los sedimentos marinos contaminados y materiales de dragado (Nendza, 2002; Casado-Martínez et al., 2006).

La razón principal para la elección de una especie para dichos tests, depende principalmente de su distribución geográfica y su disponibilidad para recolectarlo durante todo el año. En el Norte de Europa, algunas especies de anfípodos, tales como *Corophium volutator* (Pallas, 1766), han sido validadas y ampliamente utilizados para evaluar diferentes tipos de contaminación, mientras que en el Sur de Europa hay un conocimiento mucho menor de la idoneidad de especies para su utilización en tests de sensibilidad, como paso previo para poder usar dichas especies para evaluar la contaminación. Por lo tanto, en el Atlántico Sur de Europa, así como a lo largo de la costa mediterránea, existe la necesidad de realizar un mayor esfuerzo en la validación de los tests de toxicidad.

Las zonas de estuario son ecosistemas únicos, debido a los fuertes gradientes físico-químicos, tales como la salinidad, temperatura, pH, oxígeno disuelto, potencial redox, y la cantidad y composición de las partículas (Chapman y Wang, 2001). Los continuos cambios en estos parámetros puede repercutir en cambios en la biodisponibilidad de contaminantes (por ejemplo, Marín-Guirao et al., 2007). Por lo tanto, los sistemas de estuario se consideran que tienen dinámicas de biodisponibilidad de contaminantes únicas, que no son comparables con la mayoría de ecosistemas en los que los parámetros ambientales son menos variables.

Muchos de los métodos utilizados por los ministerios de medio ambiente, de diferentes países, para evaluar la calidad de los sedimentos, puede que no sean eficaces para evaluar correctamente la contaminación; y ofrezcan resultados equivocados para ese tipo de ecosistemas. Por lo tanto, existe una clara necesidad de adaptar las técnicas específicas de evaluación ambiental para las zonas de estuario (Chapman y Wang, 2001).

Por lo que respecta al impacto de la acuicultura, los principales residuos derivados de la cría de peces marinos, es la materia orgánica vertida al medio en forma de pienso no consumido, o deshechos metabólicos de los peces (Focardi et al., 2005). La recuperación bentónica apenas ha sido estudiada y la evaluación del impacto de esta actividad, con pocas excepciones (por ejemplo, Hargrave et al., 2008; Kutti et al., 2008), ha sufrido de la falta de un enfoque holístico. Los estudios se han centrado generalmente en la medición de cambios físico-químicos, así como de los parámetros de las comunidades de la macrofauna, en uno o pocos lugares, sin tener en cuenta los resultados desde una perspectiva más amplia, como las consecuencias para el funcionamiento de los ecosistemas o inferir umbrales para los deshechos generados por esa actividad (Sanz-Lázaro y Marín, 2008).

Muchos de los procesos que actúan sobre el material de deshecho, y que podrían afectar significativamente a las condiciones del bentos y confundir las predicciones, como el efecto de los peces silvestres (no cultivados), no son simulados en los modelos actuales (Chamberlain y Stucchi, 2007). Aunque el papel de los peces silvestres, en la reducción de la carga de materia orgánica particulada de la piscicultura ya ha sido descrito (Vita et al., 2004; Felsing et al., 2005), la reducción en el aporte de carbono y nutrientes por parte de los peces silvestres en las granjas de peces comerciales aún no ha sido cuantificado.

La depredación y la sucesión, son procesos ecológicos importantes que deben ser estudiados más a fondo, a fin de conseguir una mejor comprensión y capacidad de previsión, de cómo estos procesos están influenciados por diferentes niveles de enriquecimiento orgánico.

Es necesario que todas estas cuestiones mencionadas sean estudiadas, si queremos tener un mejor conocimiento de la contaminación por enriquecimiento de materia orgánica. Esto nos ayudará a reducir sus efectos y mejorar la gestión de las actividades que los producen.

Los objetivos de esta tesis son los siguientes:

- Evaluar la toxicidad de los PAH fenantreno, fluoranteno y pireno en los anfípodos *Gammarus aequicauda*, *Gammarus locusta* y *Corophium multisetosum*.

- Testar la validez de una metodología para evaluar la contaminación en sedimento de sedimentos marinos (tests de toxicidad con anfípodos y análisis de la estructura de la comunidad) en dos zonas de estuario.

- Evaluar los vertidos de carbono y nutrientes, derivados de la acuicultura de peces y sus tasas de dispersión, correlacionarlo con los parámetros físico-químicos del sedimento y la estructura de la comunidad bentónica.

- Cuantificar el efecto de los peces silvestres, como sumidero de los residuos particulados de la acuicultura de peces.

- Estudiar el papel de la depredación, sobre el sistema bentónico afectado por las piscifactorías.

- Estudiar la recuperación bentónica, en las diferentes etapas de sucesión después del cese de la actividad de producción acuícola.

ESTRUCTURA DE LA TESIS

La presente tesis consiste en cinco artículos, cada uno de los cuales constituye un capítulo. Los capítulos 1, 2 y 5 ya se han publicado, y los capítulos 3 y 4 están listos para ser enviados para su publicación. Estos documentos se han publicado, o se espera que se envíen para su publicación en revistas científicas incluidas en el SCI (ISI).

Chapter 1	Sanz-Lázaro C, Marin A, Borredat M (2008) Toxicity studies of polynuclear aromatic hydrocarbons (PAHs) on European amphipods. *Toxicology Mechanisms and Methods* 18, 323-327
Chapter 2	Sanz-Lázaro C, Marin A (2009) A manipulative field experiment to evaluate an integrative methodology for assessing sediment pollution in estuarine ecosystems. *Science of the Total Environment* 407, 3510-3517
Chapter 3	Sanz-Lázaro C, Belando M. D., Marin A, Marín-Guirao L (in prep) Linking horizontal and vertical waste dispersion with benthic impact in an offshore finfish farm with a highly efficient feeding system
Chapter 4	Sanz-Lázaro C, Belando M. D., Marin A (in prep) The role of predation in the benthic system influenced by a finfish farm with a moderate level of organic matter input
Chapter 5	Sanz-Lázaro C, Marin A (2006) Benthic recovery during open sea fish farming abatement in Western Mediterranean, Spain. *Marine Environmental Research* 62, 374-387

En el capítulo 1, los efectos de los hidrocarburos aromáticos polinucleares (PAH) derivados del petróleo, fenantreno, fluoranteno y pireno, fueron testados con los anfípodos *Gammarus aequicauda*, *Gammarus locusta* y *Corophium multisetosum*. Estas especies están ampliamente distribuidas a lo largo del Atlántico y el Mediterráneo de la zona Sur de las costas europeas. La toxicidad de estos compuestos se cuantificó mediante tests de toxicidad de laboratorio sólo con agua, obteniendo la concentración letal a las 48 horas (LC_{50}).

En el capítulo 2, se puso a prueba una metodología común para evaluar, la contaminación en sedimento de sedimentos marinos (tests de toxicidad de anfípodos y análisis de la estructura de la comunidad) en dos zonas de estuario europeas (Ría de Aveiro, Portugal, La Manga, España). Se llevaron a cabo experimentos manipulativos de campo usando tres niveles de concentración de petróleo crudo, para comparar los cambios resultantes en la estructura de la comunidad, con los tests de toxicidad con anfípodos en laboratorio e in situ con especies de anfípodos nativas *Corophium multisetosum* (zona atlántica) y *Microdeutopus gryllotalpa* (zona mediterráneo). Los impactos del contaminante se compararon en la estructura de la comunidad y en los tests de toxicidad, que se correlacionaron con la concentración de crudo.

En el capítulo 3, se realizó un estudio integrador, en una granja de peces marinos en mar abierto en el Mediterráneo Occidental, a través de la combinación de la cuantificación de las tasas de sedimentación de residuos particulados con el estado químico y biológico del bentos. Las tasas de sedimentación y dispersión de: carbono orgánico y nitrógeno particulado y el fósforo total, se midieron mediante el uso de trampas de sedimentación. Además, se cuantificó el efecto de los peces silvestres en los deshechos particulados. Se midieron los parámetros químicos de los sedimentos, así como, la estructura de la comunidad de macrofauna. La dispersión de los residuos particulados, se correlacionó con los parámetros físico-químicos y de la estructura de la comunidad macrobentónica.

En el capítulo 4, se estudiaron las consecuencias de la estructura trófica bentónica, debido a alteraciones por enriquecimiento orgánico. Mediante la colocación de jaulas de exclusión de depredación, de diferentes tamaños de malla en el fondo marino debajo de una piscifactoría, los depredadores fueron excluidos secuencialmente. Con el fin de comprobar los efectos sobre el sistema bentónico, se midieron los parámetros químicos y macrofaunísticos, los cuales son buenos indicadores del estado bentónico.

En el capítulo 5, se estudió la recuperación bentónica después del cese de la actividad piscicultora. Se midió un conjunto de diferentes parámetros químicos y biológicos en el sedimento. Además, se aplicó el índice bentónico marino Azti (AMBI). Con el fin de comprobar, si hubo cambios en el funcionamiento del ecosistema en las diferentes fases de sucesión, se estudiaron las variaciones en la abundancia de los grupos tróficos de la comunidad de la macrofauna.

CONCLUSIONES

Capítulo 1:

Gammarus. aequicuada, *Gammarus locusta* y *Corophium. multisetosum* son firmes candidatos, a ser utilizados en futuros experimentos en la evaluación de la contaminación de sedimentos por petróleo.

Capítulo 2:

El uso de una metodología integrando, los tests de toxicidad con anfípodos y análisis de la estructura de la comunidad, pueden ser considerados como una herramienta fiable para evaluar y monitorear la contaminación de petróleo en zonas de estuario.

Capítulo 3:

Los peces silvestres pueden desempeñar un papel importante, en los flujos de deposición de deshechos particulados, derivados de la acuicultura actuando como sumidero.

El alcance del impacto de la acuicultura sobre la comunidad bentónica, puede subestimarse si se evalúa teniendo en cuenta, sólo los datos de las tasas de dispersión de los residuos (medidos directamente o obtenidos a partir de modelos).

Los fondos de algas calcáreas de vida libre, son muy sensibles al impacto producido por la acuicultura comparado con los fondos sin comunidades vegetales. El umbral obtenido, fue sólo un 35% mayor que la tasa de deposición basal de carbono orgánico particulado.

Capítulo 4:

Bajo condiciones de enriquecimiento moderado de materia orgánica, los principales depredadores epibentónicos no son los peces demersales, sino especies pequeñas de invertebrados.

Estos depredadores epibentónicos parecen alimentarse, preferentemente, de depredadores de la macrofauna bentónica, y determinan que los ecosistemas bentónicos, sean más dados a tener una estructura trófica de tres niveles.

La depredación epibentónica, en estas condiciones de enriquecimiento orgánico moderado, disminuye la diversidad de la macrofauna, dando lugar a un fondo marino más reducido.

Capítulo 5:

Las etapas intermedias de la sucesión, no se pueden predecir con precisión, debido a la singularidad de los parámetros ambientales, y a la particular estructura funcional de la comunidad de cada lugar

Durante la sucesión, hay diferencias en la composición de especies y abundancia, pero también en la estructura trófica.

El índice bentónico marino Azti (AMBI) demostró su validez para la evaluación de la contaminación derivada de la acuicultura, pero no permite distinguir entre las distintas fases de la sucesión.

GENERAL ABSTRACT

Organic matter enrichment, one of the most common sources of contamination, can be generated by very diverse human activities. Coastal areas are regarded as areas of high primary productivity (Viaroli et al., 1996; Underwood and Kromkamp, 1999; Chapman and Wang, 2001). They are also considered as threatened and/or declining habitats, since they tend to be more severely affected by contaminants than other ecosystems because waves and tidal movements may encourage the accumulation of toxicants. Important biogeochemical processes take place in coastal sediments, which are related with carbon and nutrient fluxes relevant on a global scale. Any pollution affecting these areas may have serious consequences for these cycles.

The ultimate fate of suspended particulate matter is to sink and settle on the sea floor, where it is incorporated into the sediment stratigraphy. Although the sources of the particulate matter that make up the benthic habitat vary spatially and temporally, with few exceptions the microbial and faunal communities of the benthos intimately depend on the productivity of the surface waters of the oceans (Solan and Wigham, 2005).

The particulate matter input from the water column to the seabed is low and benthic communities are supported by relatively minor rates of organic matter and nutrient flux. Therefore, when organic matter input rates increase, if the organic load is large enough, it can exceed the carrying capacity of the ecosystem and produce substantial changes in the chemical parameters of the sediment, as well as in the benthic communities.

Organic matter overload consumes the oxygen of the surface of the seabed, producing hypoxia, and, if the carbon deposition rate is too high, the seafloor can reach anoxic conditions. The consequence of oxygen consumption is the appearance of bacteria with an anaerobic metabolism that uses compounds other than oxygen as electron acceptors to obtain energy. The predominant bacteria of such organically enriched sediments are *Beggiatoa*, which produce sulphides as residues of their metabolism. There are other less abundant anaerobic bacteria which produce ammonium and methane.

All these by-products, especially sulphides, are toxic to the inhabiting fauna at high concentrations, and, along with the oxygen exhaustion of the pore water, may lead to the depletion of the most sensitive species, sometimes resulting in the total defaunation of the sediment (Brooks and Mahnken, 2003; Heilskov et al., 2006).

The species inhabiting the sediment, especially the macrofauna, play a major role in the process of benthic nutrient regeneration, affecting primary production by supplying nutrients directly and enhancing rates of pelagic recycling (Grall and Chauvaud, 2002). Some of these species produce bioturbation of the sediment. Basically, this process consists of moving and mixing the sediment, thereby redistributing the particles, as a result of different activities, such us motion, feeding, tube construction, etc. The results of bioturbation may vary substantially from simple to very complex structures such us galleries, hollows or tubes of many different types, depending on the bioturbating traits of the species involved (Aller and Aller, 1998).

Sediment bacteria play a key role in the biogeochemical cycling of nutrients. The bioturbation of macrofauna may impact the diversity, structure and function of such bacteria (Findlay et al., 1990) and, in turn, have a significant effect on many ecosystem processes (Biles et al., 2002). Such activity greatly increases the sediment area exposed to water from the surface, which is able to flush into deeper layers of the seabed, where there is a low microbial activity. This process will increase carbon oxidation of anaerobically refractory organic matter (Kristensen, 2001), resulting in increased nutrient release, which will support bentho-pelagic primary production, thus enhancing benthic production (Solan and Wigham, 2005).

Since the bioturbation potential of the infauna depends on the abundance, biomass and particular bioturbating trait of each species, any decrease in the macrofauna (partial or total), may reduce the bioturbation activity and therefore fauna-mediated irrigation, subsequently decreasing benthic production. Above the natural particulate organic input levels of a given benthic ecosystem, the amount of organic load is positively correlated with narrowing of the redox potential discontinuity (RPD) layer (the depth from the seabed surface down to where an environment with oxygen persists), and if organic input is great enough, all the sediment up to its surface can be left in anoxic conditions (Pearson and Rosenberg, 1978).

Therefore, under organic enrichment impact, important changes occur in the sediment, not only in accordance with changing chemical (oxygen diminution and stimulation of anaerobic sediment metabolism) and biological (community succession to one with more opportunistic species) parameters, but also related with important functions of the ecosystem, such as nutrient recycling and organic matter mineralization.

However, in some types of organic enrichment pollution, organic matter is associated with highly toxic substances, such as PAHs (polyaromatic hydrocarbons) in petroleum. Due to their high toxicity, these compounds generate changes in ecosystems even at lower amounts of contaminant. Substances such as oil, generate direct toxicity, and not just derived from the consumption of oxygen. This fact may cause different responses in the affected ecosystems, compared with “ordinary” organic matter not containing highly toxic substances.

Toxicity tests are widely used in oil contamination, and bioassays using crustacean amphipods have become a benchmark for characterizing contaminated marine sediments and dredged material (Nendza, 2002; Casado-Martinez et al., 2006).

The main reason for choosing a test species mainly depends on their geographical distribution and their year-round availability. In North Europe, some amphipod species, such us *Corophium volutator* (Pallas, 1766), have been broadly validated and used for assessing different types of pollution, while in South Europe there is a much less knowledge of using species in sensitivity tests as a prior step to evaluate pollution. Therefore, in the Southern Atlantic as well as along the Mediterranean coast there is need for a greater effort to be applied to toxicity test validation.

Estuaries are unique ecosystems due to the strong physico-chemical gradients, such us salinity, temperature, pH, dissolved oxygen, redox potential, and the amount and composition of particles (Chapman and Wang, 2001). Continuous changes in these parameters may result in changes in contaminant bioavailability (e.g. Marin-Guirao et al., 2007). Therefore, estuarine systems are expected to have unique toxicant bioavailability dynamics, not comparable with many other ecosystems in which environmental parameters are less variable.

Many of the methods used by environmental agencies worldwide for assessing the sediment quality may not properly assess pollution and provide misleading results. Hence, there is a clear need to tailor specific assessment techniques for estuarine environments (Chapman and Wang, 2001).

As regards, to aquaculture impact, the main residue derived from marine finfish farming is the organic matter released to the environment in the form of uneaten feed or fish metabolic wastes (Focardi et al., 2005). Benthic recovery has been barely studied and impact assessment of this activity, with few exceptions (e.g. Hargrave et al., 2008; Kutti et al., 2008), has suffered from the lack of a holistic approach. Studies have usually focused on measuring changes in physico-chemical as well as macrofaunal assemblage parameters in one or few locations without considering the results in a broader perspective, such as the consequences for ecosystem functioning or inferring thresholds for exported wastes (Sanz-Lázaro and Marin, 2008).

Many processes that act on the waste material, and which could significantly influence benthic conditions and confound predictions, such as the wild fish effect, are not simulated within present day models (Chamberlain and Stucchi, 2007). Although the effect of wild fish on reducing particulate organic matter load form fish farming has already been reported (Vita et al., 2004; Felsing et al. 2005), carbon and nutrient input reduction by wild fish on commercial fish farms has not been yet quantified.

Predation and succession are important ecological processes which need to be more thoroughly studied in order to have a better understanding and forecasting ability of how these processes are influenced by different organic enrichment levels.

All the above mentioned issues need to be studied if we are to have a better knowledge of organic matter enrichment contamination. This will help us to reduce its effects and better manage the activities that produce it.

The objectives of this thesis were to:

- To evaluate the toxicity of the PAH phenanthrene, fluoranthene and pyrene to the amphipods *Gammarus aequicauda*, *Gammarus locusta* and *Corophium multisetosum*.

- To test the validity of a methodology to evaluate sediment pollution in marine sediment (amphipod toxicity tests and community structure analysis) in two estuarine areas.

- To evaluate carbon and nutrient fish farm outputs and dispersion rates, correlating them with sediment physico-chemical parameters, and benthic community structure.

- To quantify the effect of wild fish as a sink of aquaculture particulate wastes.

- To study the role of predation on the benthic system affected by fish farms.

- To study benthic recovery in different stages of succession after fish farm activity ceases.

THESIS STRUCTURE

The present thesis consists in five papers, each of which constitutes a chapter. Chapters 1, 2 and 3 have already been published, and chapters 4 and 5 are ready to be sent for publication. These papers have been published, or are expected to be sent for publication, in scientific journals included in the SCI (ISI).

Chapter 1	Sanz-Lázaro C, Marin A, Borredat M (2008) Toxicity studies of polynuclear aromatic hydrocarbons (PAHs) on European amphipods. *Toxicology Mechanisms and Methods* 18, 323-327
Chapter 2	Sanz-Lázaro C, Marin A (2009) A manipulative field experiment to evaluate an integrative methodology for assessing sediment pollution in estuarine ecosystems. *Science of the Total Environment* 407, 3510-3517
Chapter 3	Sanz-Lázaro C, Belando M. D., Marin A, Marín-Guirao L (in prep) Linking horizontal and vertical waste dispersion with benthic impact in an offshore finfish farm with a highly efficient feeding system
Chapter 4	Sanz-Lázaro C, Belando M. D., Marin A (in prep) The role of predation in the benthic system influenced by a finfish farm with a moderate level of organic matter input
Chapter 5	Sanz-Lázaro C, Marin A (2006) Benthic recovery during open sea fish farming abatement in Western Mediterranean, Spain. *Marine Environmental Research* 62, 374-387

In chapter 1, the effects of the polynuclear aromatic hydrocarbon (PAH) derivates from oil, phenanthrene, fluoranthene and pyrene, were tested with the amphipods *Gammarus aequicauda*, *Gammarus locusta* and *Corophium multisetosum*. These species are widely distributed along the Southern Atlantic and the Mediterranean European coasts. The toxicity of these compounds was quantified in laboratory toxicity water only tests by obtaining the 48 h lethal concentration (LC_{50}).

In chapter 2, a common methodology to evaluate sediment pollution in marine sediment (amphipod toxicity tests and community structure analysis) was tested in two European estuarine areas (Ria de Aveiro, Portugal; La Manga, Spain). Manipulative field experiments were conducted at three oil concentration levels, to compare resulting changes in community structure with laboratory and *in situ* amphipod toxicity tests carried out with native amphipod species *Corophium multisetosum* (Atlantic area) and *Microdeutopus gryllotalpa* (Mediterranean area).The impacts of the toxicant were compared in the community structure and toxicity tests, both of which were correlated with oil concentration.

In chapter 3, an integrative study was performed in an offshore marine finfish farm in the Western Mediterranean by merging particulate waste sedimentation rates with chemical and biological benthic status. Particulate organic carbon and nitrogen, and total phosphorus sedimentation rates and dispersion were measured in a marine fish farm, through the use of sedimentation traps. In addition, the effect of wild fish on the particulate wastes was quantified. Chemical parameters of the sediment, as well as, the macrofaunal community structure was measured. Aquacultural particulate waste dispersion was correlated with physico-chemical parameters and the macrobenthic community structure of the seabed.

In chapter 4, the consequences of benthic trophic structure alterations due to organic enrichment were studied. By anchoring predation exclusion cages of different mesh sizes on the seabed below a marine finfish farm, predators were sequentially excluded. In order to test the effects on the benthic system, chemical and macrofaunal parameters which are good indicators of the benthic status were measured.

In chapter 5, benthic recovery was studied after the cessation of fish farming. A different set of chemical and biological parameters was measured in the sediment. The Azti Marine Benthic Index (AMBI) was also applied. In order to test whether there were changes in the ecosystem functioning during the different stages of succession, the variations in the trophic group abundance of the macrofaunal community were studied.

CONCLUSIONS

Chapter 1:

Gammarus. aequicuada, *Gammarus locusta* and *Corophium. multisetosum* are firm candidates to be used in future experiments in oil sediment pollution assessment.

Chapter 2:

The use of a methodology integrating amphipod toxicity tests and community structure analysis may be regarded as a reliable tool for assessing and monitoring oil pollution in estuarine areas.

Chapter 3:

Wild fish may play an important role in aquaculture particulate waste deposition fluxes by acting as a sink.

The extent of fish farm impact on the benthic community might be underestimated if it is assessed by only taking into account data (directly measured or from models) from waste dispersion rates.

Unattached coralline algae beds are highly sensitive to fish farm impact compared to unvegetated beds. The threshold obtained was just 35% greater than the basal particulate organic carbon deposition rate.

Chapter 4:

Under moderate organic mater enrichment conditions, the main epibenthic predators are not demersal fish, but small invertebrate species.

These epibenthic predators seem to feed preferably on infaunal predators, and determine whether the benthic ecosystem is more likely to have a three-level trophic structure

Epibenthic predation, under these moderate organic enrichment conditions, decreases macrofaunal diversity, leading to a more reduced environment.

Chapter5:

Intermediate succession stages can not be accurately predicted due to the uniqueness of the environmental parameters and the singular community functional structure of each location.

During succession there are differences in species composition and abundance but also in trophic structure.

The Azti Marine Benthic Index (AMBI) proved its validity for assessing aquaculture pollution but did not distinguish between successional stages.

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CHAPTER 1

Toxicity Studies of Polynuclear Aromatic Hydrocarbons (PAHs) on European Amphipods

Abstract

The effect of phenanthrene, fluoranthene, and pyrene in dimethyl sulfoxide on the amphipods *Gammarus aequicauda*, *Gammarus locusta,* and *Corophium multisetosum* was tested in a static exposure in sea water. The 48-h lethal concentration (LC_{50}) of phenanthrene was 173.85 µg L^{-1} for *G. aequicauda*, 147.64 µg L^{-1} for *G. locusta,* and 215.20 µg L^{-1} for *C. multisetosum*. The 48-h LC_{50} of fluoranthene was 49.99 µg L^{-1} for *G. aequicauda*, 42.71 µg L^{-1} for *G. locusta,* and 2.85 µg L^{-1} for *C. multisetosum*. The 48-h LC_{50} of pyrene was 73.49 µg L^{-1} for *G. aequicauda*, 60.78 µg L^{-1} for *G. locusta,* and 25.29 µg L^{-1} for *C. multisetosum*. Together with their wide distribution along European coasts, the evidence of toxicity on the tested PAH compounds in these amphipods make these species appropriate candidates for evaluating oil-contaminated sediments in Europe.

INTRODUCTION

The bioassay using crustacean amphipods has become a benchmark in the characterization of contaminated marine sediments and dredged material (Casado-Martinez et al., 2006) and the lethal test involving amphipods is a standard technique that is frequently recommended and applied (Nendza, 2002). This approach is routinely used to measure the biological effects of sediment samples, and standard operating procedures already exist and are used by environmental protection agencies in several countries (Environment Canada, 1992; RIKZ, 1999; USEPA, 1994).

Before governments can include toxicity tests in their environmental pollution policies, these tests must be validated. Such validation should be performed by making sensitivity tests with different reference substances, depending on the nature of the toxicant that is to be evaluated and by calibrating the response of the species to these reference substances.

The choice of a particular test species will mainly depend on their geographical distribution and year-round availability. The fact that in Europe there is a marked environmental gradient between the northern Atlantic, the southern Atlantic, and the Mediterranean coasts means that different species occupy the same type of niche in each one. In northern Europe, some amphipod species, such as *Corophium volutator* (Pallas 1766), have been broadly validated and used for assessing different types of pollution, while in southern Europe there is a much less knowledge concerning which species are suitable for use in sensibility tests for evaluating pollution. Therefore, in the southern European Atlantic as well as in the Mediterranean coasts, there is a need for greater effort related to toxicity test validation.

Few oil-derived substances have been tested with amphipods from southern Europe as a prior step to evaluating sediment oil pollution. Oil is a common constituent in coastal pollution, especially in dredged and harbor sediments. Oil sediment pollution constitutes a potential threat to the coastal environment (Dauvin, 1998; Peterson et al. 2003) and to important economic activities, such as ecotourism and fisheries (Commendatore et al., 2000). Due to the variability of its chemical composition, oil is not suitable for use as a reference substance in toxicity tests, while phenanthrene, fluoranthene, and pyrene, polynuclear aromatic hydrocarbons (PAHs) derived from oil, have a constant chemical composition and therefore represent a suitable approach for the initial toxicity assessment of oil.

The amphipods used in this work are benthic species with differing distributions along European coasts. *Gammarus locusta* (L 1806) is widely distributed along the European Atlantic coast (Neuparth et al., 2002; Riba et al., 2003), while *Gammarus aequicauda* (Martinov 1931) is widespread in the Mediterranean. *C. multisetosum* (Stock 1952) is distributed along the southern European Atlantic coast, occupying the same niche as *C. volutator* in the North.

G. aequicauda has been used for assessing metal contamination in sediment (Cesar et al., 2004; Prato et al., 2006) in which it presented a 96-h lethal concentration (LC_{50}) value for cadmium ranging from 0.26 (0.24–0.18) to 5.16 (7.35–3.57) mL L^{-1} (Prato et al., 2006) and a 48-h LC_{50} value for ammonium chloride (NH_4Cl), a commonly used reference substance, of 49.68 (38.44–60.92) mL L^{-1} (Cesar et al., 2002). *G. locusta* has been used in whole sediment contamination bioassays (Costa et al., 2005; Neuparth et al., 2005) and the effects of sediment geochemical properties on copper toxicity to this amphipod have been investigated (Correia and Costa, 2000). Studies on biomarkers of copper exposure and toxicity have also been carried out using this species (Correia et al., 2002a, 2002b, 2002c; Costa et al., 2002). *G. locusta* showed a 96-h LC_{50} value for cadmium of 0.89 (0.81–0.89) mL L^{-1} and a 96-h LC_{50} for copper of 0.3 (0.17–0.52) (Costa et al., 1998). *C. multisetosum* has been used in toxicity tests involving whole sediment assessment (Casado-Martinez et al., 2006; Castro et al., 2006) as well as in perylene spiked sediment exposure (Cunha et al., 2006). However, no LC_{50} data were reported for this species in any of these works. In this study we evaluated the toxicity of the PAHs phenanthrene, fluoranthene, and pyrene on the amphipods *Gammarus aequicauda*, *Gammarus locusta,* and *Corophium multisetosum* to assess the suitability of using them in sediment oil pollution toxicity tests.

METHODS

Collection, Holding, and Acclimation of Test Organisms

The amphipod *G. aequicauda* (Martinov 1931) was collected from saline coastal lakes in Santa Pola Natural Reserve, SE Spain (Mediterranean coast), which is an unpolluted area (Cesar et al., 2004). The amphipods *G. locusta* (L 1806) and *C. multisetosum* (Stock 1952) were collected from Ria do Aveiro in Canal do Mira, N.W. Portugal (Atlantic coast), which is also considered an unpolluted zone (Castro et al., 2006). All amphipods were collected using a 0.5-mm sieve and placed in polyethylene buckets containing algal species and water. Large predators, such as crabs, carnivorous Polychaeta, and other amphipod species larger than 2 cm, were discarded. The amphipods were immediately transported in constant temperature containers to the laboratory, where they weremaintained in 20-L glass aquaria with filtered natural sea water (Whatman GF/C, 0.45 μm) under constant aeration. Their food supply consisted of Purina Rabbit Chow and Tetra-Min fish food (mixed 1:1). The amphipods were gradually acclimated to test conditions for 72 h. During acclimation, the dissolved oxygen concentration, pH, salinity, and temperature were controlled following USEPA recommendations (USEPA, 1994).

Sensitivity Tests

The toxic substances used in this study were pyrene (Fluka, >97%), phenanthrene (Fluka, >97%), and fluoranthene (Sygma, 99% HPLC), which are all PAHs derived from oil. Dimethyl sulphoxide (DMSO, Panreac, PA-ACS), was used as solvent to solubilize the PAHs in water because of its low toxicity (Hallare et al., 2006; Marquis et al., 2006; Okumura et al., 2001).

Sensitivity tests were performed in covered 1-L glass cylindrical vessels, which were previously washed with phosphate-free Extran detergent (Merck) and rinsed with distilled water. The glass vessels were then rinsed three times with acetone (Panreac, PA-ACS) and left in an acid bath. Finally, the vessels were rinsed with ultrapure water (Milli-Q).

PAH concentrations were prepared by diluting each compound from a stock solution with DMSO, so the concentration of DMSO was always constant for each tested concentration. Water control, DMSO control, and five concentrations were made for each compound using data from range-finding tests: phenanthrene 100, 160, 210, 260, 320 (µg/L); fluoranthene 30, 65, 95, 125, 180 (µg/L); and pyrene 4, 10, 20, 35, 60 (µg L^{-1}). If higher or lower concentrations were needed, dilutions were always prepared with the relevant control. Each treatment had five replicates (except fluoranthene and phenanthrene tests for *G. aequicauda*, where four replicates were performed) using glass vessels containing 800 mL of sea water. The control water and dilution water used in the experiments consisted of natural marine water (salinity of 35) collected from an unpolluted area and filtered through a GFC Whatman (0.45 µm) filter. All treatments, except the seawater control, had the same concentration of DMSO (1 mL L^{-1}).

Tests were run in constant conditions (temperature 18 ºC, light intensity 200 µE/m^2/s^1 and photoperiod 14:10-h light:dark) in a culture chamber (ASL–Snijders). All tests were of 48 h duration, which is the same as Cesar et al. (2002) used with *G. aequicauda*. No food was added during the tests. The organisms selected were males and females from 2 to 5mm in length, avoiding egg-bearing females. Each replicate contained 10 individuals. The number of survivors in each chamber was determined at the end of the exposure period. The test parameters and conditions are given in Table 1.

At the beginning and end of every test the overlying water quality parameters, including temperature, salinity, dissolved oxygen, and pH, were measured to ensure the acceptability of the tests, following standard methods (APHA 1995). Tests were carried out following the guidelines of adapted protocols of ASTM (1997) and USEPA (1994). To be considered acceptable for tests, all water and DMSO controls had to show more than 80% survival.

Statistical Analysis

To ensure that the solvent had no effect, the DMSO and water controls were compared for each substance and species of amphipod through a two-sample *t*-test. A *p* value of 0.05 or less was regarded as being significant for all the tests. Prior to the *t*-test, the normality and equality of variances were checked using Kolmogorov-Smirnov and Levene tests, respectively. The 48-h LC_{50} was calculated using Probit Analysis (Minitab, v14 software).

RESULTS

The compounds were highly toxic for all three amphipod species. Phenanthrene presented the lowest toxicity and fluoranthene the highest toxicity for all three species tested. This was particularly evident in the case of *C. multisetosum*, while *G. aequicauda* and *G. locusta* showed broadly similar sensitivities to all the toxicants tested, although, of the two, *G. locusta* was slightly the more sensitive. *C. multisetosum* showed greater tolerance to phenanthrene (Table 2). During the duration of the laboratory tests, the amphipods were observed every 12 to 24 hours. It was noticed that, before dying, the amphipods gradually lost their mobility: first, their swimming ability decreased, and then they only produced horizontal movements. Finally, only antennae and mouthparts showed some movement until they reached the point when no motion was perceived.

The sensitivity tests results pointed to water qualities within the standard range. In all the tests, the DMSO control did not show significant differences from the water control (*t*-test, $p < 0.05$). In all the controls survival was greater than

DISCUSSION

The toxicity resulting from the combination of test substance and solvent should be evaluated carefully, and the magnitude of interaction taken into consideration when comparing toxicity data from various sources (Calleja and Persoone, 1993). In this study, the solvent (DMSO) was not toxic to the species of amphipod at the concentration used in the tests (1 mL L^{-1}), which agrees with other findings of other studies in which DMSO was used with amphibians, zebrafish, and microalgae (Hallare et al., 2006; Marquis et al., 2006; Okumura et al., 2001).

Table 1. Parameters and conditions of the test using crustacean amphipods in the laboratory following USEPA recommendations (USEPA, 1994).

Parameters	**Conditions**
Test type	Static; sensitivity test
Temperature	18 ºC
Salinity	35 psu
Photoperiod	14:10 h light:dark
Light intensity	200 µE $m^2 \cdot s^{-1}$
Solvent used	Dimethyl sulphoxide (DMSO)
Test chambers	1 L glass, cylindrical and covered vessels
Volume of water	800 ml
Water used in the tests	Unpolluted water filtered through a GFC Whatman (0.45 µm) filter
Water renewal	No
Tested organisms	Males and females that ranged between 2 to 5 mm length, avoiding egg-bearing females
Number of organisms per chamber	10
Number of replicates	4 – 5
Feeding regime	No
Aeration	Constant, before and during the test
Water quality parameters	Dissolved oxygen concentration, pH, salinity and temperature were measured at the beginning and at the end of the test
Test duration	48 h
Endpoint	Survival
Test acceptability	80% survival in the water and solvent control

Our results showed that the toxic effects on *G. aequicauda* and *G. locusta* were similar, both strongly differing from *C. multisetosum*. This fact is important if further studies related to oil-polluted sediment are performed using these species. *G. locusta* and *G. aequicauda* inhabit the alga, plant, and sediment surface, while *C. multisetosum* is a scavenger. Differences in *in situ* test responses of these species may not only be due to differences of niche occupation, but also to the differing sensitivity of the species to oil compounds.

Fluoranthene toxicity values have been assessed in other species. *Ampelisca abdita* and *Rhepoxynius abronius* both showed a 24-h LC_{50} higher than 100 µg L^{-1} (Werner and Nagel, 1997), while *Corophium insidiosum* and *Rhepoxynius abronius* showed 96-h LC_{50} values of 85 µg L^{-1} and more than 70 µg L^{-1} respectively (Boese et al., 1997). In the case of pyrene and phenanthrene, no comparable results were found. Although the LC_{50} exposure times, as many other variables (solvent used, protocol modifications, etc.) in the works mentioned above, differed from those we used, rough comparisons are sufficient to affirm that the toxicity results for fluoranthene in the species tested in this experiment are within the range of those obtained in other amphipod species, which showed similar or slightly higher toxicity.

Table 2. Medial lethal concentrations [LC50s (µg L^{-1})] of phenanthrene, fluoranthene, pyrene and cadmium to the amphipods *Gammarus aequicauda*, *Gammarus locusta*, *Corophium multisetosum* and *Corophium volutator*. Values in parentheses are 95% confidence limits.

Species	Substance	LC_{50}	Reference
Gammarus aequicauda	Phenanthrene	173.85 (157.02-190.48) ^{A}	This study
	Fluoranthene	49.99 (42.23-57.48) ^{A}	This study
	Pyrene	73.49 (65.55-82.38) ^{A}	This study
	Cadmium	from 260 (240-180) to 5160 (7350-3570) ^{B}	(Prato et al., 2006)
Gammarus locusta	Phenanthrene	147.64 (136.31-158.30) ^{A}	This study
	Fluoranthene	42.71 (33.92-50.67) ^{A}	This study
	Pyrene	60.78 (54.46-67.97) ^{A}	This study
	Cadmium	890 (810-890) ^{B}	(Costa et al., 1998)
Corophium multisetosum	Phenanthrene	215.20 (204.03-226.63) ^{A}	This study
	Fluoranthene	2.85 (1.86-3.92) ^{A}	This study
	Pyrene	25.29 (22.41-28.77) ^{A}	This study
Corophium volutator	Cadmium	9030 (6150–13260) ^{B}	(Bat et al., 1998)

^{A} duration of the test was 48 h
^{B} duration of the test was 96 h

Table 2 shows the sensitivity to cadmium found in the bibliography for the species used in this experiment as well as the results of this study, except for *C. multisetosum*, for which no data related to cadmium could be found in the bibliography. Taking into account the above-mentioned possibility of comparing toxicity results from different experiments and bearing in mind the different time exposures (48 h in our experiment and 96 h in the others), we conclude that the sensitivity of *G. aequicauda*, *G. locusta,* and *C. volutator* to cadmium is much lower than to the three substances tested in this experiment.

In developing a marine estuarine sediment bioassay protocol, a number of properties are desirable for the species used as bioindicators, such as (a) broad salinity tolerance; (b) high sensitivity to common sediment contaminants; (c) high survival rate under control conditions; (d) occupation of microhabitats at or preferably below the sediment–water interface to ensure maximum and consistent exposure to sediment contaminants; (e) low sensitivity to natural sediment variables, such as particle size and organic content, to allow a wide variety of sediment types to be tested; (f) broad geographic range to enhance the breadth of its application as a test species; (g) ease of collection, handling, and maintenance in the laboratory; (h) ecological importance in estuarine systems; and (i) the ability to be cultured or year-round availability the field (Bat et al., 1998). Because they meet most of the mentioned characteristics and also show high sensitivity to the oil derivatives assayed in this paper, we consider *G. aequicuada*, *G. locusta,* and *C. multisetosum* firm candidates for use on the assessment of oil-polluted sediments.

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CHAPTER 2

A manipulative field experiment to evaluate an integrative methodology for assessing sediment pollution in estuarine ecosystems

Abstract

The assessment of sediment contamination is of crucial importance for the management of estuarine ecosystems. Environmental risk assessment of oil pollution must be specific to these ecosystems because of their unique toxicant bioavailability dynamics, which is not comparable with that of other ecosystems where the environmental parameters are less variable. The goal of this work was to test in two European estuarine areas (Ria de Aveiro, Portugal; La Manga, Spain) whether the common methodology used to evaluate sediment pollution in marine sediment (amphipod toxicity tests and community structure analysis) is suited to these physico-chemically unique systems. Manipulative field experiments were conducted at three oil concentration levels, to compare resulting changes in community structure with laboratory and in situ amphipod toxicity tests carried out with native amphipod species *Corophium multisetosum* (Atlantic area) and *Microdeutopus gryllotalpa* (Mediterranean area). The impact of the toxicant was reflected in the community structure and toxicity tests, both of which were correlated with oil concentration. These results point to this methodology being a reliable tool for assessing and monitoring pollution in estuarine areas.

INTRODUCTION

Estuaries are regarded as areas of high primary productivity (Viaroli et al., 1996; Underwood and Kromkamp, 1999; Chapman and Wang, 2001). They are also considered as threatened and/or declining habitats, since they tend to be more severely affected by contaminants than other ecosystems because waves and tidal movements may encourage the accumulation of toxicants. Petroleum and its derivatives constitute a threat to the coastal environment (NRC, 1985; Dauvin, 1998; Peterson et al., 2003) and are among the most frequently found toxicants in polluted estuarine areas. Nevertheless, unlike open rocky or sandy coasts, many estuaries are relatively protected from strong winds and currents. Hence, they tend to gather fine grained sediments, which, in tidal flats and marshes, tend to accumulate and bind toxicants that are discharged. This is the blueprint for major ecological disasters, since these intertidal areas are prime spawning and nursery grounds for many invertebrates and fish. The result may be the alteration of benthic communities, which play an essential role in the functioning of marine ecosystems, particularly in shallow coastal areas (Beukema et al., 1999). Therefore, the assessment of sediment pollution is of crucial importance for the management of estuarine ecosystems (Moreira et al., 2006).

Estuaries are unique ecosystems due to the strong physico-chemical gradients, such us salinity, temperature, pH, dissolved oxygen, redox potential and the amount and composition of particles. Among these parameters, salinity is the most important factor, controlling the partitioning of contaminants between sediments and overlying or interstitial water (Chapman and Wang, 2001). Continuous changes in these parameters may result in changes in contaminant bioavailability (e.g. Marin-Guirao et al., 2007). Therefore, estuarine systems are expected to have unique toxicant bioavailability dynamics, not comparable with many other ecosystems in which environmental parameters are less variable.

Many of the methods used by environmental agencies worldwide (e.g. U.S. EPA, Environment Canada) for assessing the sediment quality rely on benthic community structure assessment (Cesar et al., 2008). The above mentioned stressful environmental conditions also result in particular community characteristics, such as low diversity and low species richness, with few dominant species (McLusky and Martins, 1998; DelValls et al., 1998). Therefore, in this kind of environment, classical tools frequently used in the evaluation of community status may not properly assess pollution and provide misleading results.

Much attention has been paid to the monitoring and risk assessment of industrialized coastal areas exposed to contaminants from very different sources and many protocols have been developed. However, estuarine sediments cannot be treated as either fresh or marine sediments, and neither can they be properly assessed without understanding estuarine variability and processes. Hence, there is a clear need to tailor assessment techniques specifically for estuarine environments (Chapman and Wang, 2001).

Toxicity tests using amphipod crustaceans have become a benchmark for the assessment of pollution in estuarine and marine sediments (USEPA, 1994; ASTM, 1997; Nendza, 2002; Casado- Martinez et al., 2006). Both laboratory and in situ toxicity tests have been widely proven acceptable for risk assessment and each method has its own advantages and limitations. Laboratory bioassays are easy to perform and inexpensive and are carried out under more controlled conditions, while in situ toxicity tests reproduce a range of potentially relevant environmental factors that laboratory bioassays lack. However, in situ bioassays do have drawbacks; for example, they are more expensive and difficult to carry out, especially in zones where scuba diving is necessary. Nevertheless, in situ bioassays reflect ecological reality and can account for cumulative and synergistic effects, which is why they are used as part of integrative assessment studies to facilitate the taking of environmental decisions for specific impacted systems (Adams, 2003; Burton et al., 2005).

Despite the increased emphasis on using both in situ and laboratory bioassays, their ecological relevance has not been determined empirically in controlled cause effect experiments and so it is uncertain whether the results of acute toxicity tests reflect adverse population and community-level effects (Ingersoll et al., 1997). In relation to this point, a large-scale study was performed along U.S. coastlines using independent data from laboratory sediment toxicity tests and measures of benthic community structure (Long et al., 2001). In spite of the inevitable variability in the different sampling sites of this study, it could not be concluded that inter-site differences in the composition of the fauna were due to differences in the concentration of contaminants or to some other, perhaps unidentified, covariable (Morrisey et al., 1996).

Manipulative field experiments overcome this problem by establishing direct cause–effect relationships. Since the aims of environmental monitoring and ecological risk assessment are to detect and/ or predict adverse chemical impacts on populations, communities and ecosystems (Forbes et al., 2006), the establishment of any relationship between disturbance and benthic assemblages in the field is a first and necessary step towards understanding environmental impact (Underwood and Peterson, 1988). Accordingly, the use of epidemiological approach is becoming a powerful method for increasing the certainty concerning causal claims (Adams, 2003). Such methods consist of validating results by weight of evidence, using various lines of evidence independently, rather than using individual lines of evidence.

Since bioassays should be ecologically representative, the species used in our toxicity tests were the amphipods *Corophium multisetosum* (Stock, 1952) and *Microdeutopus gryllotalpa* (A. Costa, 1853). These species were chosen following the recommendations of Chapman and Wang (2001) and Calow (1989). Both are estuarine species that inhabit the sediment and are considered “keystone species”, since they sustain the ecological integrity (structure and productivity) of their ecosystems (Drake and Arias, 1995; Re et al., 2007). Additionally, they are widely distributed along European coasts (see Drake and Arias, 1995 and cites therein; Re et al., 2007; Kluijver and Ingalsuo, 2007a,b; Kluijver and Ingalsuo, 2007a,b).

Both species have been used previously in bioassays for toxicants: *C. multisetosum* mainly in bioassays relatedwith sediment quality assessment (Castro et al., 2006; Casado-Martinez et al., 2006; Cunha et al., 2006; Re et al., 2007; Sanz-Lázaro et al., 2008), and *M. gryllotalpa* for metal toxicity testing (Cesar et al., 2002; Cesar et al., 2004).

The goal of this work was to test in two European estuarine areas (Ria de Aveiro, Portugal; La Manga, Spain) whether the common methodology used to evaluate sediment pollution in marine sediment (amphipod toxicity tests and community structure analysis) is suited to these physico-chemically unique systems. Manipulative field experiments were conducted at three oil concentration levels, to compare resulting changes in community structure with laboratory and in situ amphipod toxicity tests carried out with native amphipod species *C. multisetosum* (Atlantic site) and *M. gryllotalpa* (Mediterranean site).

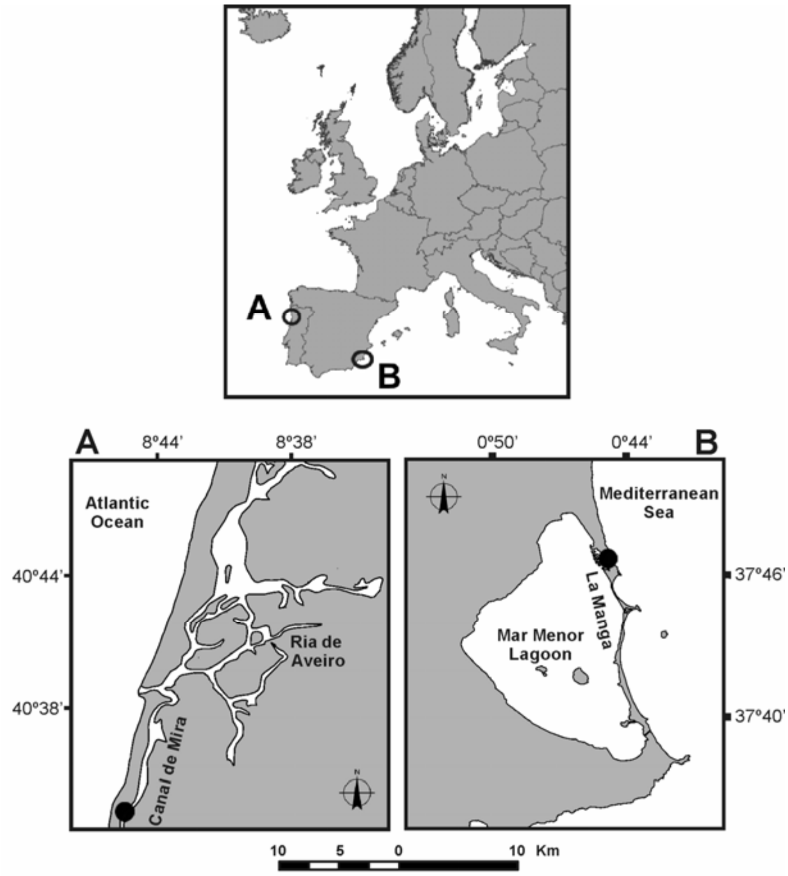
METHODS

Study area

The study was conducted in two southern European intertidal flats exposed to markedly different environmental conditions – one located on the Atlantic coast at Ria de Aveiro, NWPortugal, and the other on the Mediterranean coast, in La Manga, SE Spain (see Fig.1). Ria de Aveiro is a typical open ocean estuary and is classified as a drowned river-valley type estuary formed by the course of several rivers (Pritchard,1967). The experiment was conducted on an intertidal sand flat of the lowest part of a river entering the southern part of the Ria de Aveiro known as “Canal de Mira”, while La Manga is a sandbar that limits a coastal lagoon, the Mar Menor, which shows the typical characteristics of a semi-enclosed sea. Permanent connection to the Mediterranean is by means of a channel and several canals. The experiment was carried out in the channel, a muddy-sandy micro-intertidal flat.

In both study areas, the experiments were conducted in unpolluted zones where amphipod species abundance was high and estuarine conditions prevailed, even though these conditions (changes in salinity and tides) differed in frequency and intensity between both study areas. The variation in water parameters in the respective sites is shown in Table S1 (see the supplementary material).

Figure 1. Location of amphipod collection and *in situ* experiment sites (●). *Corophium multisetosum* was taken from Ria de Aveiro, NW Portugal (A) and *Microdeutopus gryllotalpa* was collected in La Manga, SE Spain (B).



Oil analysis

The toxicant used in these experiments was Maya crude petroleum extracted in Mexico, which is a heavy crude oil used as a reference oil for microbial degradation experiments in the context of the MATBIOPOL project (de Oteyza and Grimalt, 2004). The crude oil analysis was supplied by the analytical laboratories of Repsol IPF in Escombreras, Spain. The main chemical characteristics of the crude oil and analytical methods are shown in Table S2 (see the supplementary material).

Sediment characterization

The sediment from both study zones, as well as the sediment used as a spiked substrate, were characterized by analyzing their particle size and organic matter content. For the granulometric analysis, sediment samples were first dried at 60 °C and then separated out through a series of sieves on a mechanical shaker (Buchanan, 1984). The organic matter content was measured by weight difference, heating dry sediment at 450 °C for 5 h in a muffle furnace. Sediment type was assessed qualitatively by recording the most abundant particle size class present (Wentworth, 1922). The sediment from the study zone in the Atlantic was graded as medium sand (0.500–0.250 mm), while the sediment from the study area in the Mediterranean consisted mainly of very fine to fine sand (0.250–0.063 mm). The substrate to be spiked consisted of medium sand (0.500–0.250 mm) and was chosen because of its low organic matter content (0.7% dry weight), thus reducing the adsorption of oil to organic matter.

Collection, holding and acclimation of test organisms

The amphipods, *C. multisetosum* and *M. gryllotalpa*, were collected from the same areas where the experiments were performed in the Atlantic and Mediterranean sites, respectively.

All amphipods were collected using a 0.5 mm sieve and placed in polyethylene jars containing water from the area. Large predators were discarded. The amphipods used for laboratory testing were immediately transported to the laboratory in constant temperature containers, where they were maintained in 20 L glass aquaria containing filtered natural sea water (0.45 μm GF/C Whatman filter) under controlled conditions for acclimation. Aeration was provided and a photoperiod of 18:6 h (light:dark) was selected. Their food supply consisted of Purina Rabbit Chow and Tetra-Min fish food (mixed 1:1). The amphipods were gradually acclimated to the experimental salinity and temperature conditions over a period of 72 h, during which time the dissolved oxygen concentration, pH, salinity and temperature were monitored.

Experimental design

The sediment used as a spiking substrate was taken from an unpolluted (Mediterranean) coastal zone close to the marine reserve of Cabo de Palos-Islas Hormigas, Spain. The sediment was sieved through a 1 mm mesh to eliminate coarser particles, and any fauna removed.

Oil inoculation, spiking and stabilization were performed in accordance with USEPA (2001). Crude oil spiking was initially carried out in the laboratory, adding oil to a 0.5 L polyethylene jar containing wet homogenized spiking substrate (hereinafter referred to as “spiked substrate”). The oil and sediment were hand-shaken for 1 min and then rolled mechanically for 2 h. Afterwards, the jars were stored at 4 °C for a period of 2 to 3 days. This spiked substrate was used as the matrix to carry the pollutant, which would be used to inoculate the sediment from the study areas. Once in the field, for each replicate, the spiked substrate was mixed by hand with sediment in a polyvinyl chloride (PVC) inoculation cylinder (radius=20 cm, height=15 cm) in order to keep a constant ratio between the spiked substrate and the local sediment. Mixing was performed underwater to remove the lighter fraction of oil. For the laboratory bioassays, the mixed sediment was transported back to the laboratory immediately, while for field experiments (in situ bioassays and benthic fauna experiments) the mixed sediment was instantly poured into in situ experimental PVC cylinders (radius=20 cm, height=15 cm) anchored to the seabed. A pilot study had shown this to be an effective method for introducing oil into the sediment.

Three nominal concentration levels, low (15 mL oil kg^{-1} dry sediment), high (60 mL oil kg^{-1} dry sediment) and a control were used. The controls strictly followed the same protocol but inoculation and spiking was with water instead of oil. The experiments used a randomized design following recommendations by Hurlbert (1984). For all treatments, six replicate units were randomly distributed in zones with homogenous within-site environmental conditions (Atlantic and Mediterranean).

Toxicity tests

Both the in situ and laboratory bioassays lasted 10 days (ASTM, 1997). Ten individuals of the respective species were used in each replicate for each experiment. The amphipods were randomly selected but ovigerous females and juveniles were avoided. *C. multisetosum* was used in the in situ and laboratory toxicity tests for Ria de Aveiro, while *M. gryllotalpa* was used in the corresponding toxicity tests for La Manga. The number of survivors was determined at the end of the exposure period. Missing (decomposed) organisms and organisms that were not moving after gentle stimulation were considered dead.

At the beginning and end of the tests, water quality parameters (temperature, salinity, dissolved oxygen and pH) were measured to guarantee test validity following a standard protocol (APHA, 1995). For the in situ bioassays, water was extracted from inside the amphipod field chambers (AFC) by means of a syringe. In the in situ bioassays, the amphipods were placed, immediately after collection, in the AFCs, which consisted of PVC tubes (radius=12.5 cm, height=10 cm) closed by a 0.5mmmesh at both ends. Each AFC was placed inside an in situ experimental cylinder anchored in the sediment (see Fig. S1 in the supplementary material).

The laboratory bioassays consisted of static whole sediment tests. The test parameters and conditions are given in Table S3 (see the supplementary material). For each replicate, 200 mL of wet mixed (contaminated) sediment from the experiment areas was placed inside 1 L cylindrical glass vessels and then 600 mL of filtered seawater (0.45 µm GF/C Whatman filter) with a salinity of 35 was added. The water was added very carefully, pouring the water down the inner walls of the vessel to minimize resuspension of the contaminated sediment. After the particles had settled, the water was aerated and ten amphipods were introduced into each vessel before covering. Laboratory bioassays were kept under constant conditions (20 °C, 200 μE m^{-2} s^{-1} light intensity and 18:6 h light:dark cycle) inside an environmental chamber (ASL Snijders Sci. International S.L., Tiburg, Holland). The containers were constantly aerated and no feed was supplied. For the laboratory tests to be considered acceptable, a survival greater than 80% was deemed necessary for the control.

Benthic fauna experiments

For field experiments with benthic fauna, only in situ experimental PVC cylinders (radius=20 cm, height=15 cm) anchored in the sediment were used. The above described inoculation protocol was followed (see Experimental design) to ensure that the field assays using benthic fauna and the in situ toxicity tests were comparable. At the end of the experimental period (10 days), the whole sediment contained in the benthic fauna cylinders was washed through a 1 mm sieve. The retained sediment was fixed in a 4% buffered formalin solution, separated into major faunal groups and stored in a 70% ethanol solution for later identification. Determination of benthic groups was made to the lowest possible taxonomic level. Macrofauna ash-free dry biomass was determined separately for each species and each sample by weight difference, after drying to constant weight at 60 °C and subsequently burning at 450 °C for 5 h.

Statistical analysis

Statistical analysis used in toxicity tests

To identify significant differences between treatments in each type of toxicity test (laboratory and in situ) a one way analysis of variance (ANOVA)was performed. A *P*-value of 0.05 or lower was considered as significant for all tests. An ANOVA was performed after checking for normality with the Kolmogorov–Smirnov test and homoscedasticity with the Levene test. If the data did not meet the assumptions for parametric analysis, they were arcsin ($\sqrt{x}$+1) transformed. When significant differences were found between the treatments a Tukey post-hoc analysis ($P <$ 0.05) was performed.

Statistical analysis used in experiments with benthic fauna

ANOVA was performed to identify significant differences between treatments with regards to total community abundance and biomass, species richness, Shannon Wiener diversity and abundance of Capitellidae family (Polychaeta; a typical pollution indicator taxon) and amphipod species. If the data did not meet the assumptions for parametric analysis, they were log (x+1) transformed. When significant differences were found between the treatments, a Tukey post hoc analysis (*P* < 0.05)was performed. If, after transformation, the data still did not meet ANOVA assumptions, a non-parametric Kruskal– Wallis test was performed. When significant differenceswere found in this type of test, a Mann–Whitney test (*P* < 0.05) was applied to detect pairwise treatment differences.

Multivariate analyses were applied to the benthic fauna data using the Primer (v6) software package. Non-parametric multidimensional scaling (MDS) ordination analysis (Clarke and Warwick, 1994) was performed to examine taxa assemblage differences between treatments in order to represent the similarity between the samples. Taxa that contributed b4% to the total abundance were removed from the dataset. Then, an index of dispersion (D) was applied to all routines that showed significant evidence of clumping, and so data were dispersion-weighted following recommendations by Clarke et al. (2006). A Bray–Curtis similarity matrix (Bray and Curtis, 1957) was calculated after transforming to the fourth root. The ANOSIM routine is a multivariate non-parametric test of differences between a priori defined groups, analogous to ANOVA (Clarke, 1993). This permutation test was used to assess the significance of differences between treatments in each location separately. Following recommendations made by Clarke and Warwick (1994), the data used in this analysis were given a milder transformation (square root) than used for MDS.

RESULTS

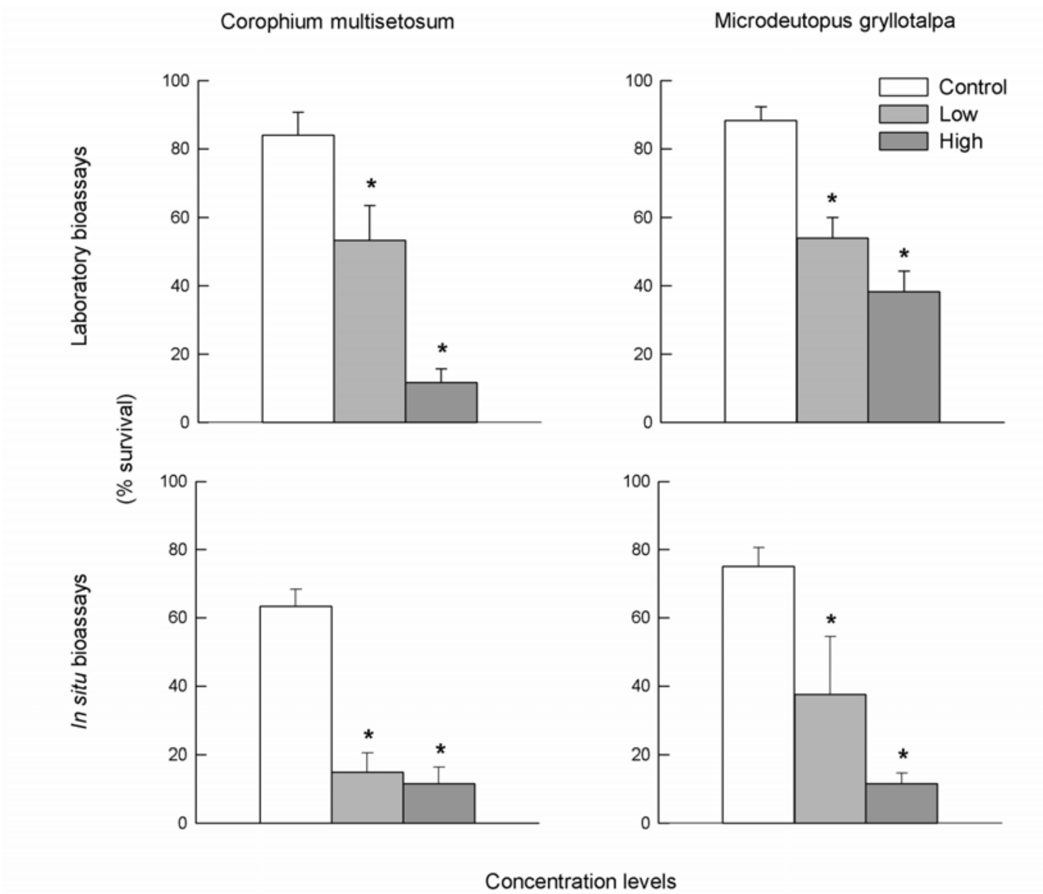
Toxicity tests

In all the toxicity tests, both laboratory and in situ and for both amphipod species (*C. multisetosum* for the Atlantic and *M. gryllotalpa* for the Mediterranean), amphipod survival decreased with increasing oil concentration in the sediment. For each test, survival was significantly different for the contaminated and non-contaminated treatments (Fig. 2).

Benthic fauna experiments

In both the Atlantic and the Mediterranean zones, acute exposure to oil-contaminated sediment caused changes in community structure. MDS analysis provided similar results for both experimental zones, with a gradient that followed oil concentration. Low oil concentrations clustered with the controls while the high concentrations formed another group.

Figure 2. *C. multisetosum* and *M. gryllotalpa* survival (mean ± SE, n=6) in 10 day laboratory and *in situ* toxicity tests in Aveiro (NW Portugal) and La Manga (SE Spain) respectively. Asterisks indicate significant differences (ANOVA, Tukey test, P < 0.05) from its respective control. Concentration levels were **control**, **low** (15 mL oil kg^{-1} dry sediment) and **high** (60 mL oil kg^{-1} dry sediment).



ANOSIM analysis showed greater differences between treatments in the Atlantic than in the Mediterranean. In the former, both contaminated treatments significantly differed from the control, while in the Mediterranean, only the higher oil concentration clearly contrasted with the control (Table 1).

In the Atlantic, total abundance and ash-free dry biomass was significantly higher in the control compared with the oil treatments, while neither species richness nor Shannon–Wiener diversity differed between treatments. In the Mediterranean, no significant differences were found in any treatment for total abundance, species richness, total ash-free dry biomass or diversity (Tables 2 and 3).

Table 1. ANOSIM (analysis of similarity) results comparing benthic fauna assemblages as a function of oil concentrations (n=6). Analysis was restricted to comparisons between treatments which were done separately for each site. Concentration levels were **control**, **low** (15 mL oil kg^{-1} dry sediment) and **high** (60 mL oil kg^{-1} dry sediment).

		Global statistics	Pairwise comparison	
			Control vs Low	Control vs High
Atlantic	*R*	0.683	0.683	0.756
(n=18)	*P*	0.001	0.002	0.002
Mediterranean	*R*	0.304	-0.063	0.343
(n=18)	*P*	0.002	0.710	0.011

In the Atlantic, *C. multisetosum* abundance was significantly higher in the control than in the oil treatments. In the Mediterranean, although *M. gryllotalpa* abundance was higher in the control than in the oil treatments, no significant differences existed between treatments. The abundance of capitellids differed between the control and the high oil concentration for both the Atlantic and the Mediterranean sites (Tables 2 and 3).

Table 2. Benthic fauna parameters (mean ± SD, n = 6) at both studied areas. Concentration levels were **control**, **low** (15 mL oil kg^{-1} dry sediment) and **high** (60 mL oil kg^{-1} dry sediment).

	Atlantic			**Mediterranean**		
	Control	Low	High	Control	Low	High
Total abundance (ind m^{-2})	3851 ± 455	959 ± 124	1201 ± 524	2092 ± 453	1816 ± 579	2735 ± 1040
Species richness	5.2 ± 0.75	4.5 ± 0.84	5.7 ± 1.03	9.5 ± 2.35	9.5 ± 1.05	9.8 ± 1.83
Total biomass (g m^{-2})	2.2 ± 0.36	1.0 ± 0.17	0.8 ± 0.29	4.3 ± 3.18	1.9 ± 0.78	2.9 ± 1.98
Shannon-Wiener diversity ($\log_2$)	1.7 ± 0.30	1.8 ± 0.30	2.0 ± 0.12	2.5 ± 0.50	2.5 ± 0.28	2.4 ± 0.33
C. multisetosum / *M. gryllotalpa* abundance (ind m^{-2})	1957 ± 572	209 ± 138	107 ± 25.5	417 ± 514	197 ± 86.7	141 ± 136
Capitellidae abundance (ind m^{-2})	0 ± 0	0 ± 0	231 ± 156	564 ± 195	440 ± 252	1286 ± 691

Table 3. ANOVA and Kruskal-Wallis results for different faunal descriptive indices for both study areas and *post-hoc* analysis results for Tukey and Mann-Whitney pairwise comparisons (n=6). Concentration levels were **control**, **low** (15 mL oil kg^{-1} dry sediment) and **high** (60 mL oil kg^{-1} dry sediment).

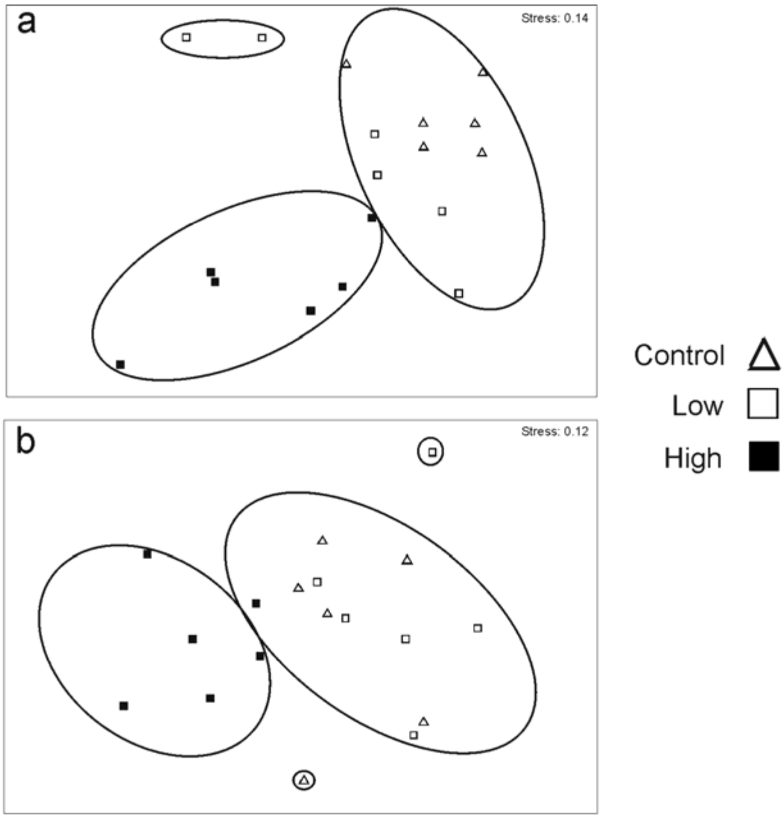
		Pairwise comparison	
	P (n=18)	Control vs Low *P*	Control vs High *P*
Atlantic			
Total abundance	< 0.001 ^{A}	<0.05	<0.05
Species richness	0.104 ^{A}	n. s.	n. s.
Total biomass	< 0.001 ^{A}	<0.05	<0.05
Shannon-Wiener diversity	0.079 ^{A}	n. s.	n. s.
C. multisetosum abundance	< 0.001 ^{A}	<0.05	<0.05
Capitellidae abundance	0.003 ^{K}	n. s.	<0.05
Mediterranean			
Total abundance	0.304 ^{A}	n. s.	n. s.
Species richness	0.936 ^{A}	n. s.	n. s.
Total biomass	0.529 ^{K}	n. s.	n. s.
Shannon-Wienner diversity	0.871 ^{A}	n. s.	n. s.
M. gryllotalpa abundance	0.143 ^{A}	n. s.	n. s.
Capitellidae abundance	0.007 ^{A}	n. s.	<0.05

^{K} = Kruskal-Wallis test
^{A} = ANOVA test
n. s. = non-significant

DISCUSSION

In this study, several lines of evidence were evaluated to establish the cause–effect relationships between the contaminant (oil) and its environmental impact in two different intertidal areas. This work comprised a manipulative field experiment, which followed the epidemiological approach suggested by Adams (2003). It established causality between the environmental stressor and the benthic status based on multiple lines of evidence: (1) coincidence of cause and effect: amphipods were seen to be sensitive in all the experiments performed in this work, as has been reported in the literature (Gesteira and Dauvin, 2000; Gesteira and Dauvin, 2005), (2) consistency of association: similar results have been found for other amphipod species (Brils et al., 2002), (3) biological gradient: there was a dose–response relationship, (4) experimental evidence: laboratory (controlled experiment) results supported the cause– effect relationship observed in the field experiments.

Figure 3. MDS plot based on the species abundance of the oil concentration levels of the benthic fauna experiments: a) in Aveiro (NW Portugal) and b) in La Manga (SE Spain). The MDS results are grouped according to the ensembles produced by the cluster results; each group has a similarity of 75 % or higher. Concentration levels were **control** (△), **low** (□) (15 mL oil kg^{-1} dry sediment) and **high** (■) (60 mL oil kg^{-1} dry sediment).



The impact of the toxicant on the estuarine ecosystems was reflected in the results for both community structure and toxicity tests, and the changes observed were comparable in all experiments performed. The greatest dissimilarity among community samples was observed between the control and the high oil concentration, while in both laboratory and in situ bioassays, toxicity increased with higher oil concentrations. However, Atlantic and Mediterranean experimental areas showed different community responses to oil pollution. According to ANOSIM, the intertidal community assemblages from the Atlantic seem to have been more affected by oil inoculation than those from the Mediterranean, especially at the low concentration level (Table 1).

Some of the classical univariate community descriptors did not detect pollution, and significant changes were only detected between control and toxicant treatments in total abundance and biomass in the Mediterranean site. In contrast, the species richness and Shannon– Wiener diversity values were similar in all treatments at both sites. This may have resulted from the low species richness and the high abundance typical of estuarine sites. In this way, although amphipods were the most sensitive organisms to oil exposure, no significant differences were found in *M. gryllotalpa* densities. The significant level of clumping [index of dispersion (D)=7.91] of this species may have masked the oil effects. In these systems, toxicant input may have an effect similar to depredation, reducing the abundance of the predominant species, which could increase the equitativity and diversity (Tables 2 and 3). Similar behaviour of the macrofauna structure has been observed by Cesar et al. (2008). Community changes were not only due to a decrease in the numbers sensitive species such as amphipods but also to the entrance of opportunistic species, such as members of the polychaete family, Capitellidae, which increased their population density in high oil concentrations. This finding agrees with Holmer et al. (1997), who reported that genus Capitella are often present in high numbers in organic-rich sediments polluted with oil components.

While the response in toxicity tests may show a linear relationship with toxicant concentration, perturbed ecosystems and communities do not usually demonstrate such straightforward behaviour. These systems are more likely to resist perturbation until a threshold is reached, above which serious changes are produced (Hyland et al., 2005). This may be the reason why the MDS analysis of both studied zones showed that benthic fauna communities were severely disturbed as pollution increased above a certain threshold, while no substantial variations were observed at low oil concentration levels. Accordingly, the most noticeable effects on community structure were detected at the high oil concentration (Fig. 3).

The outcome of risk assessment procedures will depend on the choice of species used in a bioassay. Several studies have shown that different species may show different responses to the same level of sediment contamination (Ingersoll et al., 2002; Milani et al., 2003). For this reason, the amphipods *C. multisetosum* and *M. gryllotalpa* were selected, since both are regarded as “keystone species” in their respective ecosystems and thus toxicity tests should reflect ecosystem impact. The toxicity tests with *C. multisetosum* and *M. gryllotalpa* showed differences between laboratory and in situ assays. Thus, it may be hypothesised that the effects of oil toxicity in the in situ amphipod assays were magnified by natural fluctuations in the environmental variables. Since the amphipods used for the in situ and the laboratory toxicity tests belonged to the same population, the individuals seemed to be suitable for use in the bioassays according to the results obtained in the control treatments of the laboratory bioassays.

The relatively low survival rate of the controls in the in situ toxicity tests may have been due to changes in environmental conditions, together with the effect of the amphipod field chamber. Even so, survival decreased for both species as the concentration of oil increased and, in all the bioassays, the control results were significantly different from those obtained at both concentrations of oil-contaminated sediment. The use of manipulative field experiments may be regarded as a sensitive method for testing sediment quality criteria and for defining more environmentally relevant criteria than those based solely on laboratory studies or correlative field data (Morrisey et al., 1996). Contamination studies in the laboratory do not reproduce the full range of potentially relevant environmental factors present in nature. As a consequence of these factors, the actual effect of contaminants in the field may differ from the effects identified in the laboratory. This is a major issue for intertidal flats, which are naturally stressed environments due to their dynamism (Chapman and Wang, 2001).

For a realistic estimation of sediment quality and to reduce uncertainties, the use of the "weight of evidence" framework its recommended, integrating different lines of evidence in sediment quality assessments (Chapman et al., 2002; DelValls et al., 2004). In this experiment, laboratory bioassays demonstrated direct toxic effects by the contaminant, while in situ bioassays integrated the environmental parameters from the experimental areas. Both of these independent lines of evidence suggested that the changes in benthic fauna structure were due to the toxic effects of the contaminant and allowed other covariables to be discarded as the cause of these changes. In this study, there was no need to carry out chemical quantification of the toxicants in sediment since the same nominal concentrations were loaded into the sediment at both sites. It should also be emphasised that in situ toxicity tests are time consuming and effort intensive and can be highly impractical in deep water ecosystems, such as sublitoral systems. But, given that in situ bioassays are feasible in most estuarine systems (since they are located in intertidal zones), they may be considered important as an independent line of evidence forming part of an integrative method for the above mentioned reasons.

In conclusion, the epidemiological approach used in this study, allowed us to hypothesize (by weight of evidence) that the changes produced in the community structure were due to the effects of the contaminant and not to natural variability. The aims of environmental monitoring and risk assessment are to detect and/or predict adverse chemical impacts on populations, communities and ecosystems (Forbes et al., 2006). These results suggest that this methodology may be regarded as a reliable tool for assessing and monitoring pollution in estuarine areas. Using a combination of these lines of evidence seems to be effective in this type of environment where in situ bioassays are feasible.

The most remarkable features of this approach are that it is: (1) straightforward (it does not involve the use of sophisticated equipment), (2) reliable (it uses different lines of evidence), and (3) ecologically sound (it involves the use of key-stone tests species of the ecosystem and analyzes the whole community) for pollution assessment in intertidal areas. If neither of the tested species can be found at a specific site, the methodology could be used with similar species. For example, *Corophium volutator*, which is very abundant in estuarine areas of northern Europe, has very similar ecological traits to *C. multisetosum* and has already been used in oil derived toxicity tests (Grant and Briggs, 2002; Morales-Caselles et al., 2007; Sanz-

Lázaro et al., 2008). Nevertheless, this experiment must be regarded as a starting point. The same approach should be followed with other typical toxicants from estuaries, or a mixture thereof, and in other locations before the method can be validated for broader application. After such research, the proposed methodology, along with contaminant quantification in sediments, might be recommended for assessing and monitoring sediment pollution in European estuarine systems.

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SUPPLEMENTARY MATERIAL

Table S1. Range of values obtained for various water parameters during the period of experimentation (December 2005-Febraury 2006) in both study areas (Ria de Aveiro, Portugal, and La Manga, Spain).

Parameters	Atlantic	Mediterranean
Salinity	1.40 – 6.30	37.80 – 43.38
Temperature (ºC)	6.0 – 11.7	10.8 - 15.3
pH	7.96 – 8.04	8.19 - 8 .50
Sea level variation (m)	1.00	0.30
Dissolved oxygen level in water (mg L^{-1})	8.04 – 19.50	8.50 – 13.00

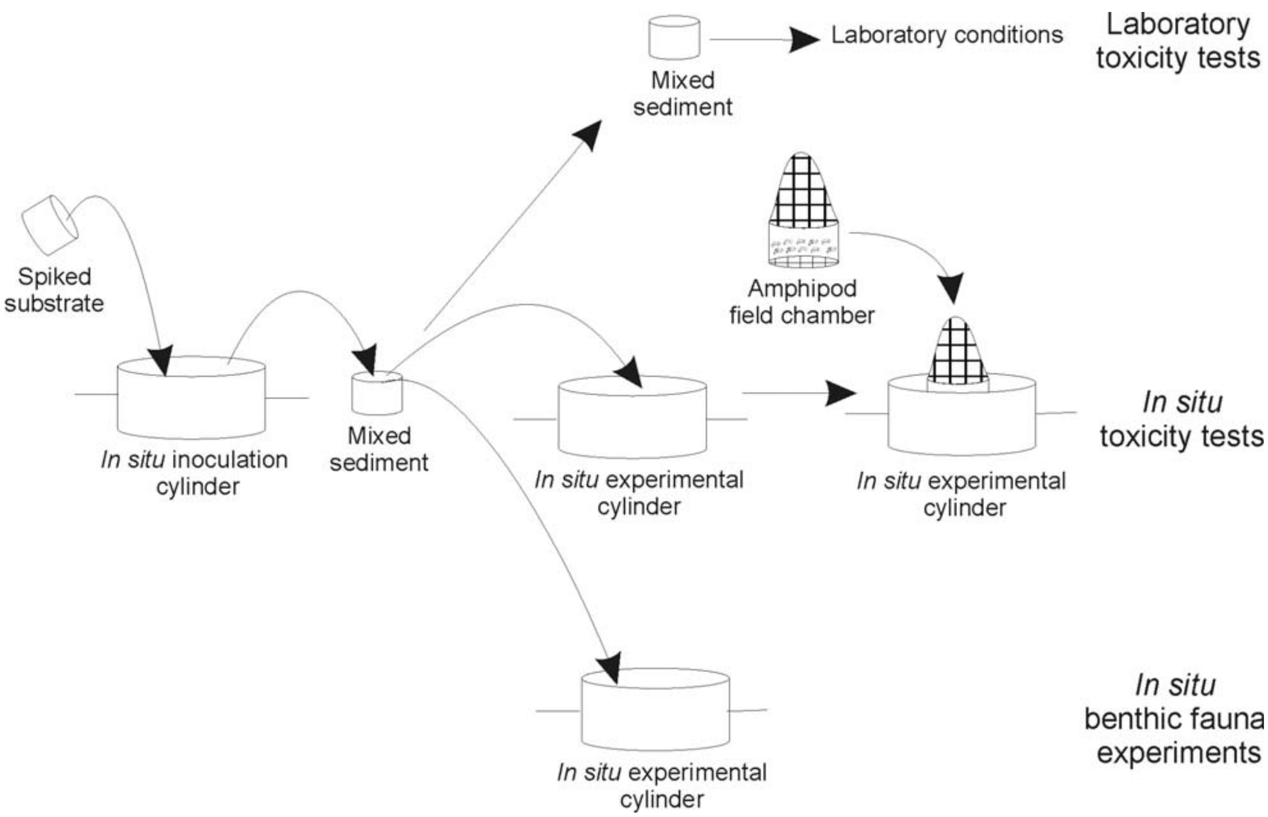
Table S2. Characteristics of the crude oil used for sediment spiking. Data provided by Repsol IPF, Escombreras, Spain.

Specification	**Value**	**Test method**
Density at 15 ºC (gr mL^{-1})	0.92	ASTM D-1298
Specific weight 15.6/15.6 ºC (g mL^{-1})	0.92	ASTM D-1298
API density (ºAPI)	21.42	ASTM D-1298
Sulphur (% wt wt^{-1})	3.14	ASTM D-4294
REID Vapour pressure (kPa)	22.80	ASTM D-323
Pour point (ºC)	-27	ASTM D-97
Viscosity at 20 ºC (cSt)	209.10	ASTM D-445
Viscosity at 40 ºC (cSt)	81.33	ASTM D-445
Dissolved sulphide (ppm vol vol^{-1})	17	ARAMCO H-3
Coal wastes (% wt wt^{-1})	11.70	ASTM D-4530
Nitrogen (ppm wt wt^{-1})	2707	Elemental analysis
Vanadium (ppm wt wt^{-1})	268	Atomic absorption
Nickel (ppm wt wt^{-1})	49	Atomic absorption
Neutralization number (mg KOH g^{-1})	0.16	ASTM D-664
Water content (% vol)	< 0.10	ASTM D-4006
PIONA analysis between 15 ºC and 77 ºC section:		
N-Paraffin content (% vol)	51.03	PIONA chromatography
I-Paraffin content (% vol)	37.77	PIONA chromatography
Naphthene content (% vol)	9.40	PIONA chromatography
Polynaphthene content (% vol)	0	PIONA chromatography
Aromatic content (% vol)	1.80	PIONA chromatography
Liquefied petroleum gas content:		
C2 content (% wt wt^{-1})	0.04	Gas chromatography
C3 content (% wt wt^{-1})	0.19	Gas chromatography
iC4 content (% wt wt^{-1})	0.10	Gas chromatography
nC4 content (% wt wt^{-1})	0.45	Gas chromatography
iC5 content (% wt wt^{-1})	0.44	Gas chromatography
nC5 content (% wt wt^{-1})	0.67	Gas chromatography

Table S3. Parameters and conditions of the test using crustacean amphipods in the laboratory.

Parameters	**Conditions**
Test type	Static; whole sediment
Temperature	20 ºC
Salinity	35
Photoperiod	18:6 h light:dark
Light intensity	200 μE m^2 s^{-1}
Volume of sediment	200 mL
Volume of overlying water	600 mL
Test chambers	1 L volume, glass, cylindrical and covered
Water used in the tests	Unpolluted water filtered through a 0.45 μm GF/C Whatman filter
Water renewal	No
Tested organisms	Organisms were randomly selected, avoiding ovigerous females and juveniles
Number of organisms per chamber	10
Number of replicates	6
Feeding regime	No
Aeration	Constant, before and during the test
Water quality parameters	Dissolved oxygen concentration, pH, salinity and temperature were measured at the beginning and at the end of the test
Test duration	10 days
Endpoint	Survival
Test acceptability	80% survival in the control

Figure S1. Schematic of the experimental design.



CHAPTER 3

Linking horizontal and vertical waste dispersion with benthic impact in an offshore finfish farm with a highly efficient feeding system

Abstract

An integrative study was performed in an offshore marine finfish farm in the Western Mediterranean by merging particulate waste sedimentation rates with chemical and biological benthic status. Particulate organic carbon (POC) and nitrogen (PON), and total phosphorus (TP) sedimentation rates and dispersion were measured. In addition, the effect of wild fish on the particulate wastes was quantified. Aquacultural particulate waste dispersion was correlated with physico-chemical parameters and the macrobenthic community structure of the seabed. The POC, PON and TP released by the fish farm was 24.98, 1.88 and 3.06 kg day^{-1} respectively. The good husbandry practices of the studied fish farm produced low feed wastage compared with other fish farms, the waste representing 0.23%, 0.11% and 1.49% of the total POC, PON and TP respectively, of the feed supplied. Wild fish also contributed to reducing the particulate organic input to the benthic system. Both facts contributed to a one order of magnitude decrease in the exported organic load compared to other fish farms. This is the first time that the wild fish effect has been quantified for its contribution to POC, PON and TP load reduction in a commercial finfish farm. The findings demonstrate that wild fish may play an important role in aquaculture particulate waste deposition fluxes by acting as a sink. TP was seen to be a sensitive indicator of exported aquaculture particulate wastes. Nevertheless, the level of fish farm impact on the benthic community might be underestimated if it is assessed by

only taking into account data (directly measured or from models) from waste dispersion rates. The seafloor was formed of unattached coralline algae beds, which produced different patterns in the community descriptors from those expected for unvegetated beds. The threshold value for this benthic ecosystem above which diversity starts to be affected was 0.087 g m^{-2} day^{-1} for POC (35% greater than the basal level). Therefore, this type of seabed is highly sensitive to fish farm impact compared to unvegetated beds. Predictive deposition models of aquaculture particulate wastes should bear in mind such issues.

INTRODUCTION

Marine aquaculture has increased substantially worldwide in recent decades and is expected to follow the same trend (FAO, 2004). The main negative impact of aquaculture is the resulting organic enrichment, which mainly consists of fish faeces and uneaten food (Focardi et al., 2005). A combination of factors, including production levels, feed characteristics and feeding efficiency, influence the quantity and quality of the material released by a fish farm in the form of fish fecal material and uneaten feed (Chamberlain and Stucchi, 2007). Such wastes take two forms: particulate and dissolved. In the water column, due to the strong diluting effect of the sea, concentrations of dissolved wastes are rapidly reduced close to background levels, whereas particulate wastes tend to sink and accumulate on the seabed. This process may produce important changes in sediment geochemistry and in the inhabiting communities.

Benthic impact assessment due to organic enrichment has been extensively studied (Pearson and Rosenberg, 1978). The spatial fate and potential effect of aquaculture particulate wastes on benthic status is site-specific and influenced by local physical chemical and biological parameters (Karakassis et al., 1999; Sanz-Lázaro and Marín, 2006). Research on benthic fish farm impact has been focused on two separate, but closely related topics, which need to be analyzed using an integrative approach. In some cases, studies have focused on predicting the reach of aquaculture particulate wastes by particulate waste dispersion modelling, while in others, aquaculture benthic impact has been directly assessed by studying chemical and biological changes in the seabed. However, most of the studies carried out to date, with some exceptions(e.g. Kutti et al., 2008; Díaz-Almela et al., 2008) cannot be considered integrative since they study isolated compartments with no attempt to merge them.

If aquaculture is to be properly managed from an environmental point of view, we must to improve our knowledge of two main points: (i) the flux of wastes exiting fish farms and reaching the seabed, and (ii) the carrying capacity of the different benthic ecosystems affected by these exported wastes. These thresholds can not be obtained by using partial/fragmented approaches (Sanz-Lázaro and Marin, 2008).

A great number of waste dispersion models have been developed to estimate the reach of organic residues derived from aquaculture. Nevertheless, robust and defensible information is not available for some of the key model parameters, such us nutrient dispersion and sedimentation rates (Islam, 2005), an area where the accuracy of the different models varies greatly (Chamberlain and Stucchi, 2007).

This lack of accuracy in models, may be due to parameter, as well as, to model uncertainty. Parameter estimates are very uncertain when sampling is limited (Higgins et al., 2003). In the case of marine finfish farm waste dispersion models, parameter uncertainty is important due to the fact that field measurements of particulate wastes are scarce and robust replication over time is lacking, especially in the Mediterranean (Holmer et al., 2007). Hence, more data related with aquacultural waste sedimentation dynamics are needed to reduce parameter uncertainty.

Model uncertainty occurs when models do not take into account parameters that play an important role in a process. Dispersion models of fish farm particulate wastes do not take into account some parameters which could be important, such as the effect of wild fish. Coastal aquaculture net pens have a strong aggregative effect for wild fish (Dempster et al., 2006). As an example, fish farms covering an area of just 1 to 4 ha may have up to 40 tonnes of wild fish around them (Dempster et al., 2004). Wild fish reduce the amount organic matter released by a fish farm, and its nutrient quality (Vita et al., 2004). Similarly, wild fish were found to consume a substantial proportion of the total particulate nutrient fluxes released by an experimental finfish aquaculture facility (Felsing et al., 2005). Therefore, the consumption of marine fish farm particulate wastes by wild fauna could be significant, and the assessment of the magnitude of this nutrient sink is important if waste loading on the seabed is to be predicted accurately (Hevia et al., 1996; Felsing et al., 2005).

To the best of our knowledge, no work has quantified the extent to which carbon and nutrient fluxes from a commercial aquaculture facility is reduced by wild fish, and no waste dispersion model has taken this effect into account. In order to establish a complete picture of how nutrients are dispersed or assimilated in coastal systems in sea-cage culture conditions, the role that wild fish play should be fully assessed and integrated into models (Dempster et al., 2005).

There is growing pressure for aquaculture to become a more ecologically sound activity (Naylor et al., 2000). Consequently, studies linking waste dispersion rates with benthic community structure must be performed to identify particulate flux thresholds below which nutrient input does not produce severe changes in benthic communities. In this way, protection agencies will be able to ensure the sustainability of aquacultural practices.

The aim of this work was: (1) to evaluate fish farm carbon and nutrient outputs and dispersion rates; (2) to correlate these outputs with the physico-chemical parameters of the sediment, and the benthic community structure and (3) to quantify the effect of wild fish as a sink of aquaculture particulate wastes.

METHODS

Study area

The study was conducted in the surroundings of a marine fish farm located in Águilas, SE Spain, (western Mediterranean; 37º 24' 56.2" N, 1º 32' 4.0" W), which produces guilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). The fish farm studied consisted of two groups of 12 net cages with an approximate constant annual production of 1000 tonnes. Each fish cage had a diameter of 25 m and the net reached a depth of 19 m. The selected fish cage was located at the edge of the fish farm facility "up-current" of the other cages, having a depth of 31 m and contained only guilthead sea bream. The depth was 31 m. During the sampling period in the studied fish cage, an average of 83,000 kg of fish was cultured and the daily feed input varied greatly: 994 ± 527 kg day^{-1} (mean ± SD).

The feeding system of the fish farm is highly optimized since it used of a floating silo, a computerized system and underwater cameras. The computerized system automatically distributes feed from the silo among the fish cages at an optimal rate and frequency, according to the fish repletion, which is controlled by the fish farm staff through the use of underwater cameras.

The feed pellets supplied to fish were cylindrical with a diameter of 6 mm. The concentration of the main elements contained in the feed is shown in Fig 1. During the study time the currents had a mean value of 0.08 m s^{-1} (Valeport 106 current meter, Valeport Limited, Dartmouth, UK; located in the fish farm next to the fish cages at a depth of 15 m). The water temperature, at a depth of 6 m, during the sampling period ranged between 26 and 21 ºC. The seabed consisted of carbonate sand with a unattached coralline algae community.

Particulate sedimentation sampling

Particulate matter fluxes, particulate organic carbon (POC) and nitrogen (PON), and total phosphorus (TP), were measured by means of sedimentation traps 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. The stem of the funnel was too narrow to allow the passage of fish. To integrate the great variability of organic wastes released by the fish farm, particulate measurements were replicated in time. Five to seven measurements, taken every 5 days, were obtained within each station during the six weeks that the experiment lasted (September and October of 2006).

Vertical waste dispersion

The bulk of particulate matter exported by the fish farm was inferred by means of the biomass cultured in the studied fish cage, the total biomass cultured in the whole fish farm and the area of all fish cages of the fish farm.

In order to measure the wild fish effect, vertical fluxes were measured by using two sedimentation traps that were placed under the net cage. One sedimentation trap was clamped beneath the net cage, to quantify the amount of wastes released by the fish farm, and the other one below the net cage close to the seabed, to measure the sedimentation rate of the wastes reaching the sediment. Water current and wild fish effects were assumed to be the variables that could cause differences between the amount of POC, PON and TP in each sedimentation trap. To distinguish both effects, the spatial extent of uneaten feed was calculated using the settling particle horizontal displacement model proposed by Gowen et al. (1994):

$$d = \frac{D \cdot V_c}{V_f} \qquad (1)$$

where D stands for the water depth between both sedimentation traps (7 m), V_c stands for the mean current velocity (0.08 m s^{-1}) and V_f stands for the settling velocity of feed particles used [0.144 m s^{-1} according to Vassallo et al. (2006)]. Taking into account that the sedimentation trap next to the seabed was placed exactly below the centre of the fish cage, if the distance obtained with the formula was less than the radius of the fish cage, it could be assumed that the variation in POC, PON and TP sedimentation fluxes would mainly be due to the wild fish effect. In this case, the wild fish effect could be calculated as a percentage of the variation in POC, PON and TP flux sedimentation between both sedimentation traps below the fish net, taking as reference the sedimentation trap clamped to the fish net, as is shown in the following formula:

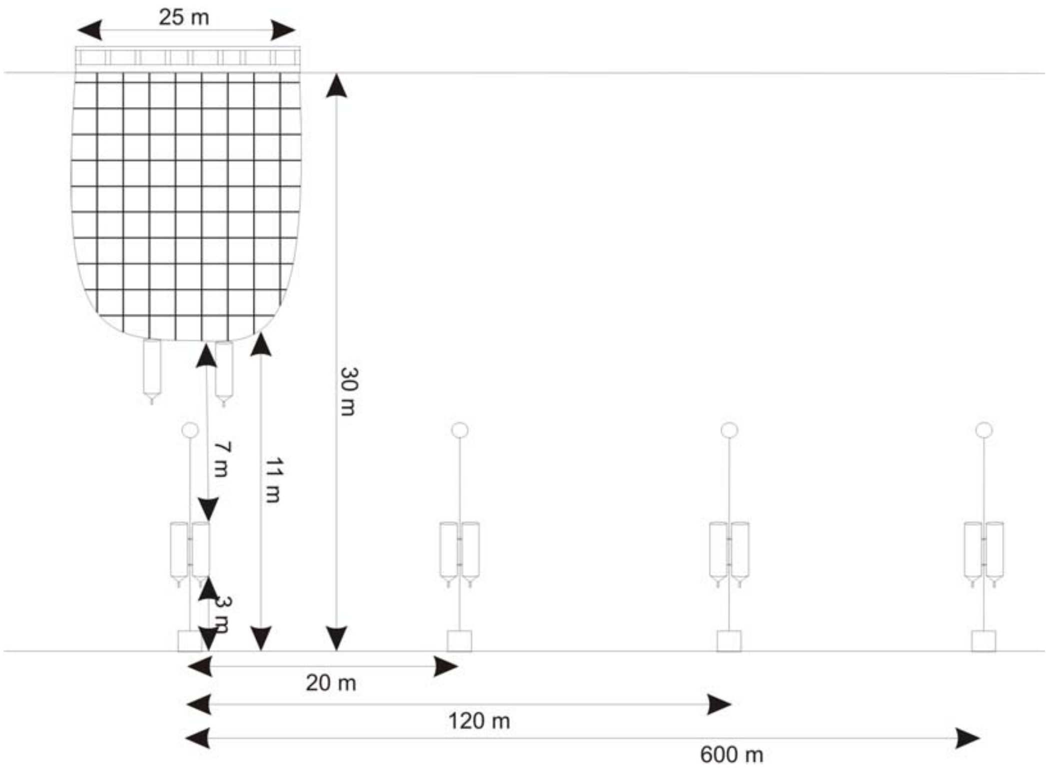
$$w = 100 - \frac{W_f \cdot 100}{W_b} \qquad (2)$$

where W_f stands for the waste flux sedimentation just beneath the fish net and N_b stands for the waste flux sedimentation close to the seabed.

Horizontal waste dispersion

Particulate waste dispersion was analyzed by measuring the POC, PON and TP sedimentation rates using the sedimentation trap placed along a spatial gradient upstream of the prevailing water-current, at increasing distances from the fish farm (0, 20, 120 and 600 m). The sedimentation traps, by means of a mooring system, were installed 3 m above the seabed to avoid resuspension (Fig. 2).

Figure 2. Arrangement of the sedimentation traps.



Physico-chemical analysis

Sedimentation trap samples were stored frozen and thawed for analysis. They were centrifuged at 4°C for 10 min at 1000 rpm and the overlying water was then carefully removed (Vita et al., 2004). The settled particles were dried in an oven at 60 ºC to a constant weight before being finely ground. Particulate organic carbon (POC; after a pre-treatment consisting of adding 1:1 HCl), and particulate organic nitrogen (PON) were determined using a Carlo Erba Inst. EA 1108 Elemental Analyser (Carlo Erba Strumentazione, Milan, Italy). Total phosphorus (TP) was determined following the 4500-PE Ascorbic acid method (APHA, 1995).

Sediment sampling

Sampling stations were located close to the sediment traps used for measuring horizontal waste dispersion (0, 20, 120 and 600 m). The seabed was sampled once during the period that the sediment traps were deployed. Four replicates were taken at each sampling station. Physico-chemical sediment parameters were sampled by divers using cylindrical hand-operated corers. The top 2-3 cm of the sediment were used for the analysis. The samples were immediately transported to the laboratory. Macrofaunal samples were taken using a hand grab (400 cm^2).

Sediment grain size was assessed by dry-sieving with a mechanical shaker through a series of sieves (2, 1, 0.5, 0.25 and 0.064 mm mesh), in accordance with the Wentworth scale (Buchanan, 1984). The organic matter content was measured by weight difference, heating previously dried sediment at 450 ºC for 5 hours. The redox potential was measured with an Orion ORP 91-80 electrode. The total ammonia nitrogen (TAN) content of interstitial water was measured with an Orion 95-12 ammonia electrode. POC, PON and PT percentage in the sediment were determined following the same protocol as used for the samples from the sedimentation traps.

Macrofauna was used as an accurate surrogate of benthic status. For the macrofaunal analysis, sediment samples were washed through a 1 mm sieve. The remaining sediment was fixed in a 4% formalin buffered solution, separated into major faunal groups and stored in a 70% alcohol solution for later identification. The determination of benthic groups was made to the lowest possible taxonomic level. Macrofauna ash-free dry biomass was determined separately for each sample by weight difference, after drying to constant weight at 60 ºC and subsequently heating at 450 ºC for 5 h.

Wild fish abundance

To estimate the abundance of dominant wild fish in each sampling station, wild fish counts were conducted at 10 and 20 m depth using an underwater camera and image analysis. Following the same methodology, a volume of 32 m^3 (4 m wide x 4 m long x 6 m deep) was analysed for each image.

Statistical analysis

The POC, PON and TP sedimentation rates and the respective concentrations in sediment, at increasing distances from the fish farm were correlated using a linear Pearson correlation, after verifying that underlying statistical assumptions were not violated (Statistica, v6).

Macrofaunal data (abundance and species richness) were used to calculate the Shannon-Wiener diversity index (*H'*). As descriptors of the community structure, macrofaunal abundance, species richness, biomass and *H'* were plotted vs distance from the fish farm. Species richness and *H'* were used as indicators of the benthic community status.

POC, PON and TP horizontal fluxes as well as community descriptors (abundance, biomass, species richness and *H'*) were regressed against distance from the fish farm. Regressions were performed using a dynamic curve fitting, basically, a non-linear curve fitting by means of an iterative process using the Marquardt-Levenberg algorithm (Marquardt, 1963). The best-fit equation to the data was a single exponential decay, three parameter equation, $f=y_0+a\ exp^{-b\ x}$ (SigmaPlot v10). When the *P*-value of the analysis of variance (ANOVA) of the whole regression was 0.05 or lower, the regression was considered significant.

ANOVA was performed to identify significant differences between stations with regard to POC, PON and TP horizontal fluxes and concentrations in the sediment, as well as, the community descriptors. For wild fish abundance, ANOVA was performed at both depths at each station. A *P*-value of 0.05 or lower was considered significant for all tests. If the data did not meet the assumptions for parametric analysis, a log(x+1) transformation was applied. When significant differences were found between the treatments a Tukey *post-hoc* analysis ($P < 0.05$) was performed. If, after transformation, the data still did not meet ANOVA assumptions, a non-parametric Kruskal-Wallis test was performed. When significant differences were found in this test, a Mann-Whitney test ($P < 0.05$) was applied to detect pairwise treatment differences.

Multivariate analyses were applied to the macrofaunal data using the Primer (v6) software package. Principal component analysis (PCA) was employed to show the relationship between the stations (samples) according to the physico-chemical parameters (variables). As a previous step to avoid skew, a log(x+1) transformation was applied to the variables, which were then normalised (subtracting the mean and dividing by the standard deviation) for the scales to be comparable. Following this, a Pearson correlation analysis between the physico-chemical parameters and the scores of the first two axes of the PCA analysis was carried out in order to ascertain the extent to which the physico-chemical parameters correlated with the two main axes of the PCA. These axes were respectively multiplied by the variance explained by each axis, and were used in the group-average clustering procedure based on Euclidean distance. SIMPROF was used to find significant differences (significance level 5%) in the classification between the samples.

A non-parametric multidimensional scaling (MDS) ordination analysis (Clarke and Warwick, 1994) was performed to examine differences in the assemblages of taxa to represent the similarity between the samples. The index of dispersion (*D*) was applied to all routines that showed significant evidence of clumping, and so data was dispersion-weighted following the recommendations by Clarke et al. (2006). A Bray–Curtis similarity matrix (Bray and Curtis, 1957) was calculated after a mild transformation (square root) following Clarke and Gorley's (2006) recommendations. After MDS, a group-average clustering procedure based on Bray-Curtis similarity and SIMPROF was applied to find differences (significance level 5%) in the classification between the samples.

RESULTS

Vertical waste dispersion

POC, PON and TP exported waste calculated for the studied cage 0.23%, 0.11% and 1.49%, respectively, of the total feed supplied and the extrapolated rate of exported POC, PON and TP by the whole fish farm was 24.98, 1.88 and 3.06 kg day^{-1} respectively.

The result of the settling particle horizontal displacement model using the mean current velocity proposed by Gowen et al. (1994) was 3.9 m. According to this formula, the maximum current velocity that allows the sedimentation trap next to the seabed to collect the feed pellets is 0.26 m s^{-1}. Less than 2% of the water current logged data were above this value. So there was almost no decrease in the amount of uneaten feed collected between the two sedimentation traps below the fish net (i.e. the sedimentation trap clamped to the fish net and the sedimentation trap close to the seabed) due to a current effect. Hence, it was assumed that the variation of POC, PON and TP sedimentation rates was only due to the wild fish effect.

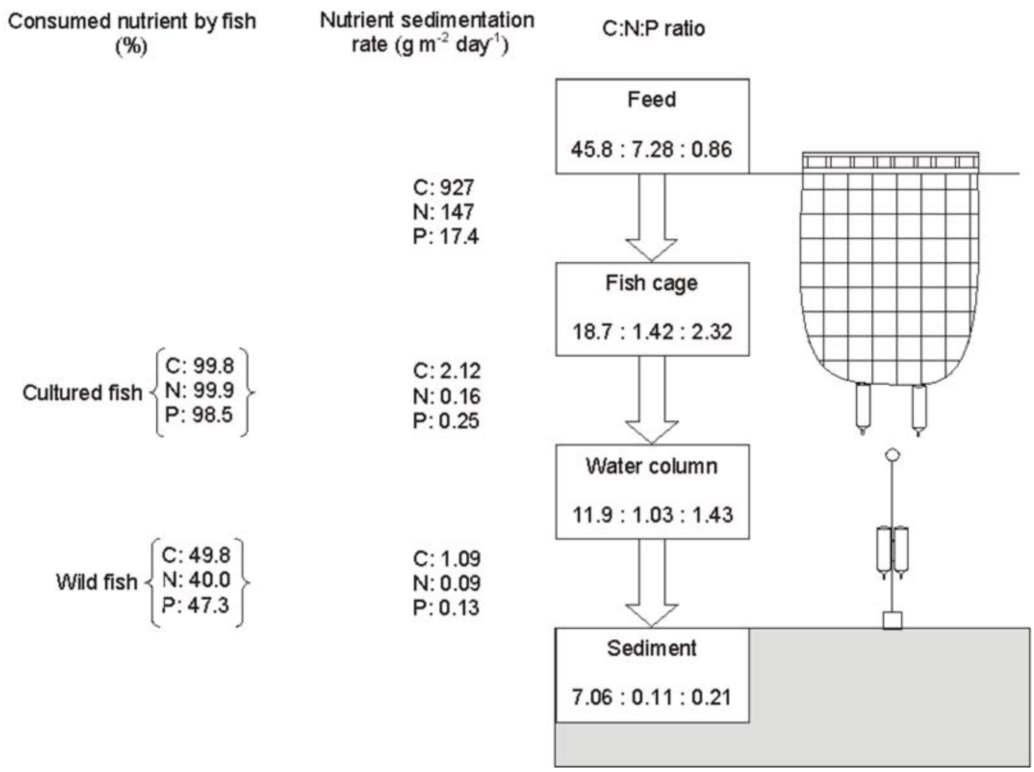
Wild fish attraction to feed was clearly noticeable. When the cultured fish were being fed in a specific cage there was a great deal of movement of wild fish in the surroundings of this cage, which was evident from the water surface. Underwater this effect was more noticeable, since the light intensity was greatly reduced, becoming close to darkness. The abundance of wild fish was significantly greater for the station beneath the fish net that in the remaining stations at both sampling depths (10 and 20 m), which demonstrated an important aggregation effect in this aquaculture facility (Table 1).

Table 1. Abundance of wild fish species per 32 m^3 at 10 and 20 m depth at sampling (mean ± SE; n=4).

	Distance from fish farm at 10 m depth (m)				Distance from fish farm at 20 m depth (m)			
	0	20	120	600	0	20	120	600
Sparidae								
Boops boops	151 ± 41	0	0	0	93 ± 31	0	0	0
Oblada melanura	6 ± 2	0	0	0	0	0	0	0
Sarpa salpa	0	3 ± 3	0	0	0	0	0	0
Carangidae								
Trachurus sp	2 ± 1	0	0	0	25 ± 18	0	0	0

The wild fish effect was quantified according to Formula 2 (see Material and Methods), and showed a maximum decrease of organic matter of 49.8 % of the total amount of POC released from the fish cage. As regards nutrients, PON and TP showed decreases up to 40.0 and 47.3%, respectively, of the total amount of PON and TP exported form the fish cage (Fig. 1).

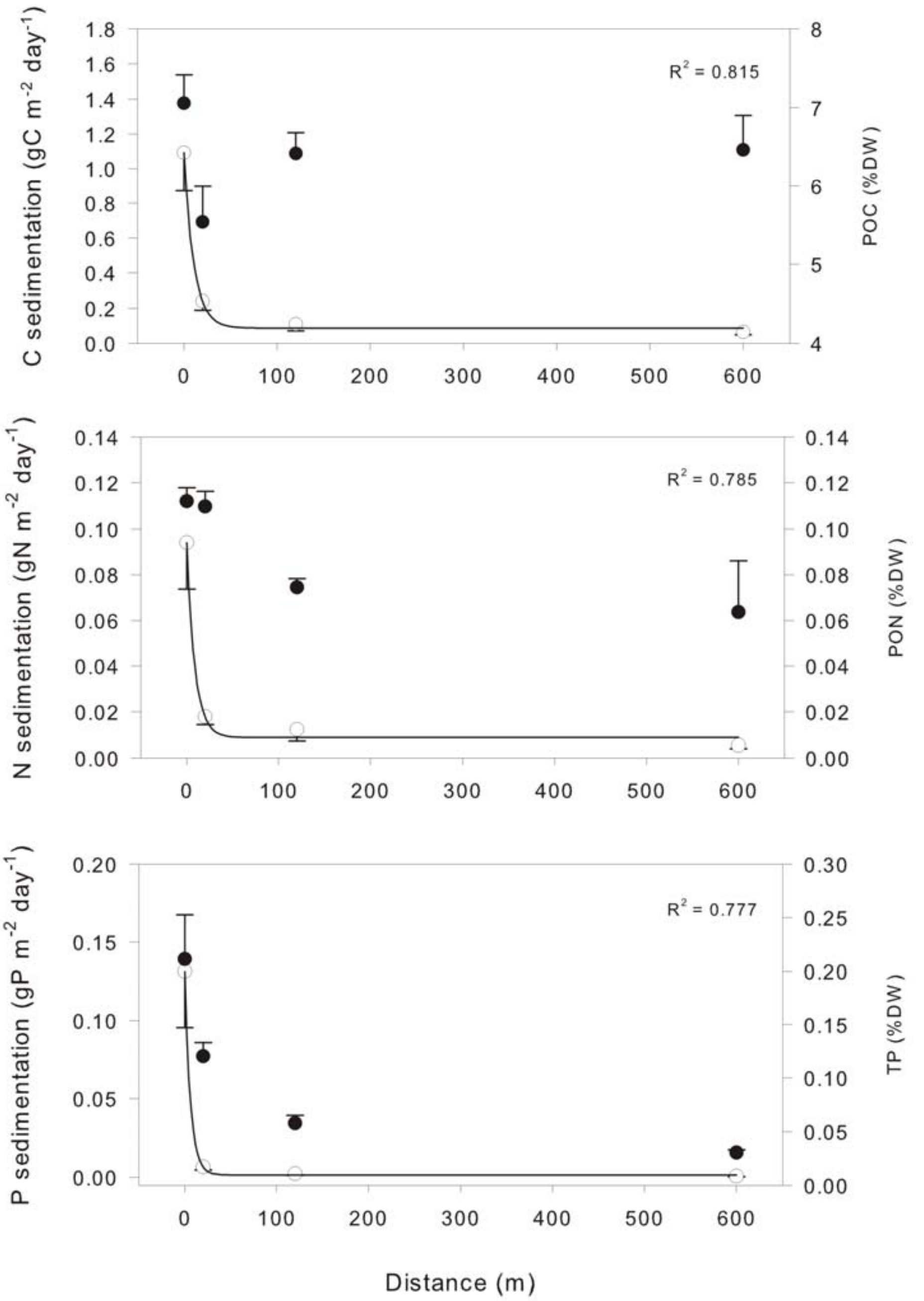
Figure 1. Diagram of vertical fluxes of particulate organic carbon, nitrogen and total phosphorus between compartments in the studied fish farm. Scheme also shows element concentration on each compartment and fish effect on particulate carbon and nutrient flux removal. Data correspond to mean values.



Horizontal waste dispersion

The regression models of the POC, PON and TP sedimentation rates in relation to distance from the fish farm were significant (Table 2). These sediment rates were one or two orders of magnitude greater below the net cage than in the furthest station. All fluxes fell exponentially as distance from the fish farm increased. POC, PON and TP sedimentation rates exhibited a great variability between replicates beneath the fish cage. This variability decreased as distance from fish farm increased (Figs. 3 and 4).

Figure 3. Particulate organic carbon (POC), nitrogen (PON) and total phosphorus (TP) sedimentation rates (open circles; mean ± SE) and concentrations in sediment surface (filled circles; mean ± SE) along the distance gradient. Solid curves show non-linear curve fitting for sedimentation rates. Overall model fit is shown in the upper right of each graph.

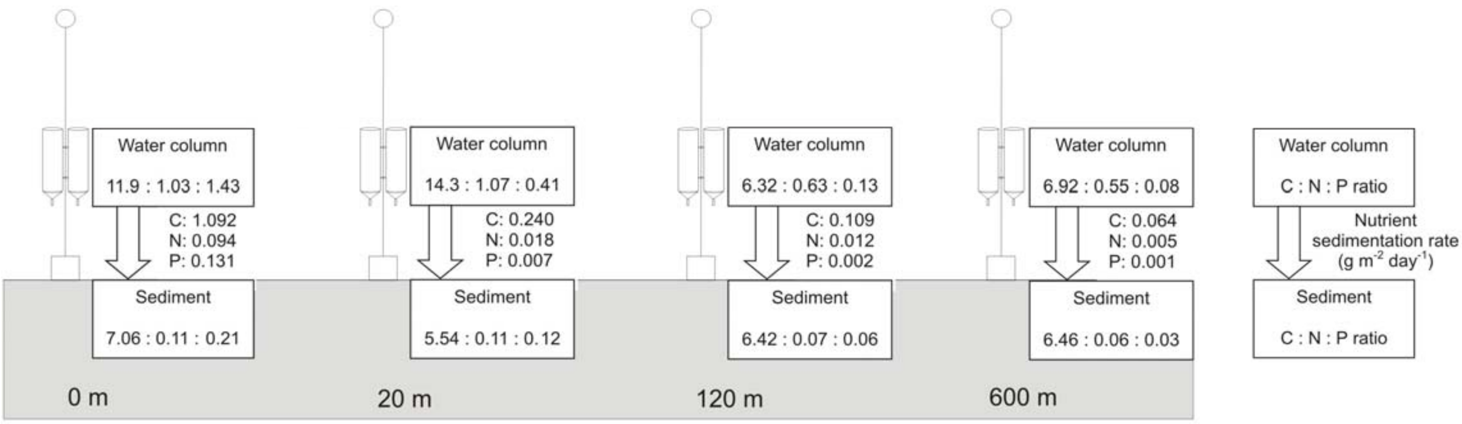


There was a positive correlation between sedimentation rates and sediment concentration of the measured parameters along the spatial gradient. Pearson correlation coefficients between sedimentation traps and sediment samples were 0.62, 0.69 and 0.90 for POC, PON and TP, respectively. TP sediment concentration was significantly greater below the fish net than in the stations situated 120 and 600 m from the fish farm. However, POC and PON sediment concentrations did not significantly differ between stations.

Table 2. Statistical significance of all non-linear regressions along the studied distance gradient from the fish farm and their coefficients. For all regressions, the best-fit equation to the data was a single exponential decay, three parameter equation, i. e. $f=y_0+a \exp^{-b\ x}$. POC, PON and TP correspond to particulate organic carbon, nitrogen and total phosphorus, respectively.

		Parameter					
		y_0		a		b	
	Regression ANOVA (*P*)	Coefficient	Significance (*P*)	Coefficient	Significance (*P*)	Coefficient	Significance (*P*)
POC sedimentation rate	<0.0001	0.0868	0.1443	1.0056	<0.0001	0.094	0.0108
PON sedimentation rate	<0.0001	0.0088	0.1169	0.0853	<0.0001	0.1124	0.0486
TP sedimentation rate	<0.0001	0.0015	0.8492	0.1299	<0.0001	0.1624	0.2848
Abundance	0.0019	2386.4	<0.0001	-1797.1	0.0005	0.0194	0.1863
Biomass	0.0464	4.2407	0.1557	-3.1943	0.0174	0.0509	0.4387
Species richness	<0.0001	31.544	<0.0001	-22.177	<0.0001	0.0229	0.0111
H'	<0.0001	4.2718	<0.0001	-1.4345	<0.0001	0.034	0.0124

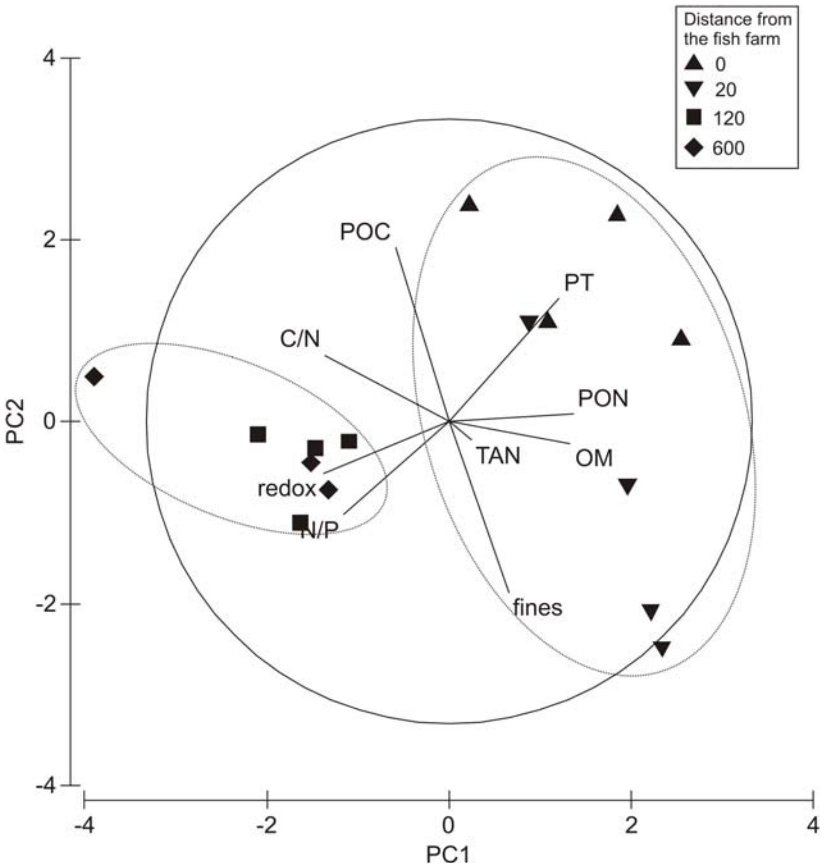
Figure 4. Diagram of horizontal fluxes of particulate organic carbon, nitrogen and total phosphorus between compartments and element concentration on each compartment along the studied distance gradient from the fish farm. Data correspond to mean values.



Physico-chemical parameters

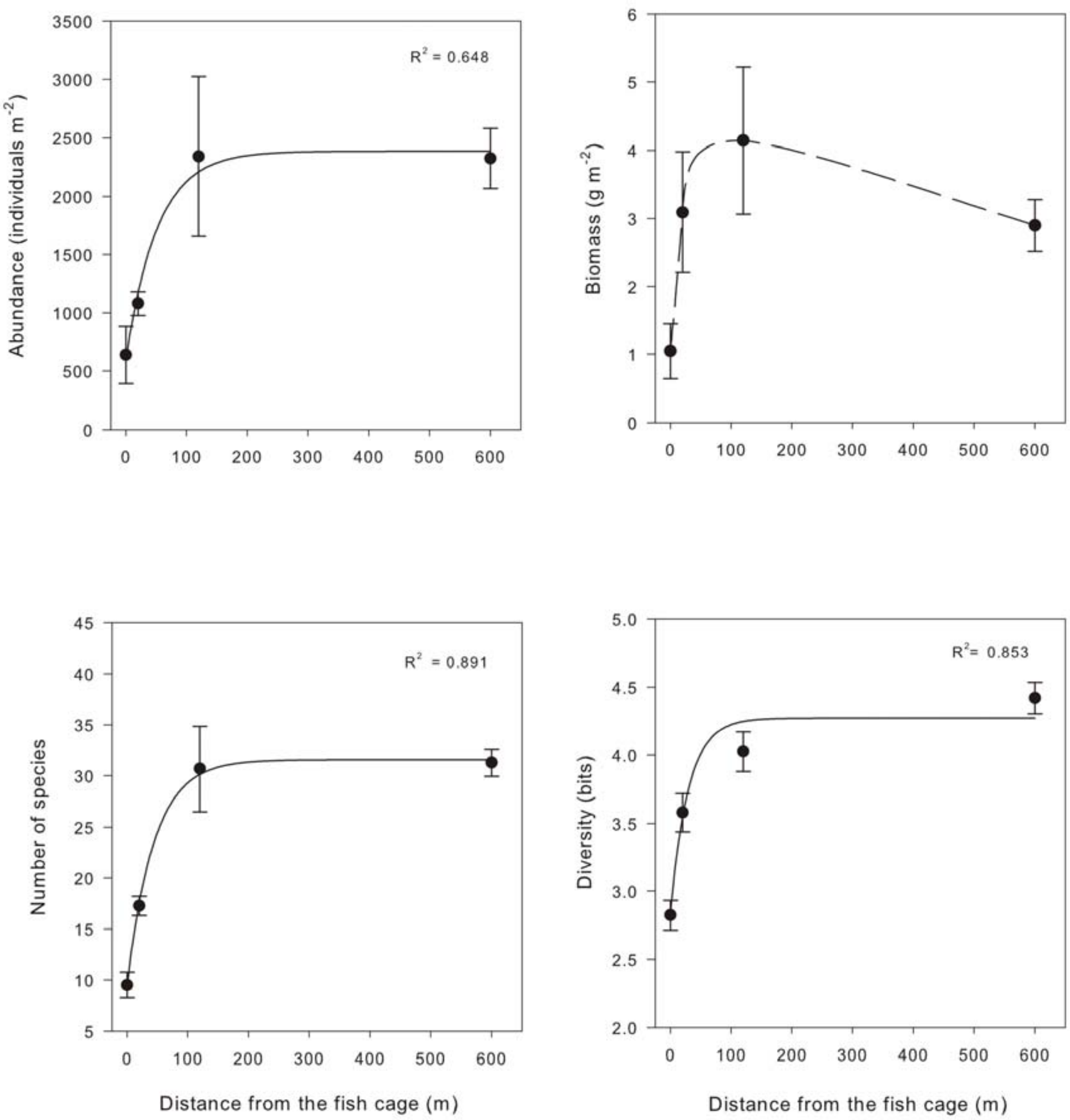
In the PCA analysis, principal components one (PC1) and two (PC2) accounted for 44.3 and 21.7 % of the variability, respectively. PC1 grouped the stations at 0 and 20 m distance from the fish farm together, and those located at 120 and 600 m. The Pearson correlation analysis between the physico-chemical parameters measured and PC1 and PC2 showed that PON (0.82), OM (0.80) and PT (0.73) were highly correlated with PC1, while redox potential (-0.83), C/N (-0.82) and N/P(-0.70) were negatively correlated. For PC2, POC (0.82) was highly correlated, while the fine granulometric fraction (-0.79) was negatively correlated.

Fig 5. Principal component analysis between stations according to the physico-chemical parameters of the sediment: fines percentage (fines), redox potential (redox), total ammonia nitrogen (TAN), organic matter (OM), particulate organic carbon, nitrogen and total phosphorus (POC; PON; TP) and the atomic ratios of nutrients (C/N and N/P). Results have been grouped according to SIMPROF (dotted lines; significance level 5%).



A cluster analysis of PC1 and PC2 using SIMPROF arranged the variables into two sets that showed significant differences between them. One group included the stations that were situated 0 and 20 m from the fish farm and the other group comprised the 120 and 600 m stations (Fig 5). In the first group, most of the samples for each station were assembled in two different groups separated by the PC2 axis, while in the second group samples from both stations were gathered into a single group.

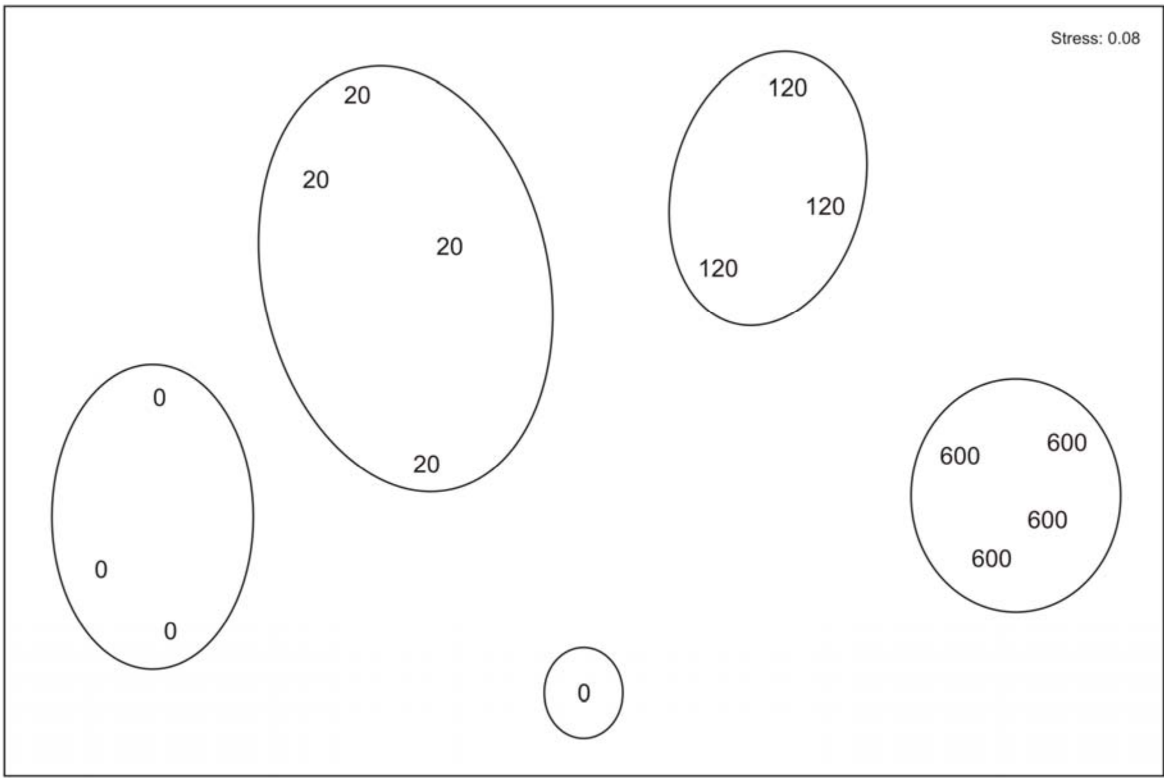
Figure 6. Descriptors of the community structure (mean ± SE) along distance gradient. Solid curves show non-linear curve fitting. Overall model fit is shown in the upper right of each graph.



Benthic structure

Coralline algae were clearly impacted in the stations located 0 and 20 m from the fish farm. The calcareous algae seemed to be dead showing a darkish colour and a noticeably smaller size compared with the more distant stations. At 120 m from the fish farm the coralline algae had a typical yellowish and pinkish pigmentation, but algal density was visibly lower than in the station located 600 m from the fish farm. This fact was reflected in the amount of chitons and other typical grazers from this ecosystem, whose densities were much lower compared with the station situated at 600 m.

Figure 7. Non-parametric multi-dimensional scaling plot of macrofaunal community abundance along the distance gradient from the fish farm (indicated by the numbers). Results have been grouped according to SIMPROF (significance level 5%). The samples from the same station have the same symbol. Arrows indicate similarity trend between groups according to MDS.



The regression models of all the community structure descriptors were significant except for biomass. Macrofaunal abundance, biomass, species richness and *H'* showed the same trend and opposite to the POC, PON and TP sedimentation rates along the spatial gradient from the fish farm. The community structure descriptors reached their asymptotic point further from the fish farm than the POC, PON and TP sedimentation rates (Fig 6).

According to the MDS plot, the effect of fish farm particulate wastes on the benthic community structure showed a gradient, which suggested that this effect was still noticeable 120 m from the fish farm. SIMPROF significantly grouped samples of each station. MDS values showed that the within-station similarity between samples increased with distance from the fish farm. Only one sample from below the fish net stations was not grouped within any other (Fig. 7).

DISCUSSION

Feed loss is a transient process within cage culture and depends upon the particular physical, biological and feeding characteristics at a farm site (Corner et al., 2006). Previous studies at other fish farms found that mean waste feed is around 5% (Chamberlain and Stucchi, 2007 and cites therein). In our study, by comparing the flux of POC between the quantity of feed input and sedimentation rates exiting the fish cage, waste was seen to be 0.23 %, which represents a decrease of one magnitude order in the amount of exported POC.

The output of particulate wastes in the studied fish farm, which used a high technology feeding system, was very low due the use of underwater cameras, a silo and a computerized system for feed distribution. The mean POC flux reaching the seabed below the fish farm was 1.09 g m^{-2} day^{-1}, while nutrient fluxes were 0.09 and 0.13 g m^{-2} day^{-1} for PON and TP, respectively. These results, considering the time of the year when the study was performed, are low compared with other works in other marine fish farms (Tsutsumi et al., 2006 and references therein; Holmer et al., 2007; Kutti et al., 2007), and suggest that substantial reductions in the aquaculture waste loads can be achieved by applying new technology to husbandry practices.

Uneaten feed and faeces are the main waste particles from fish farming activity (Focardi et al., 2005). In salmon fish farming uneaten feed can contribute up to >80% of the predicted carbon flux (Chamberlain and Stucchi, 2007). The contribution of uneaten feed close to a fish cage might even be greater for gilthead sea bream culture, since the settling velocity of feed pellets is greater for gilthead sea bream than for salmon, while the opposite applies to faecal pellets (Magill et al., 2006; Vassallo et al., 2006; Chamberlain and Stucchi, 2007). For this reason, most of the particulate material collected under the cage is expected to be feed pellets.

This study shows that wild fish play an important role in the reduction of particulate farm wastes (carbon as well as nutrients) in the fish farm studied. Similar findings have been reported in an experimental fish farm facility (with a very low cultured biomass, not representative of commercial fish farms) in Australia, where wild fish made a considerable contribution to decreasing particulate nutrient flux (40-60%; Felsing et al., 2005). In the Mediterranean, wild fish reduced 80% of the total organic matter released by a fish farm and produced notable decreases in the nutrient quality (Vita et al., 2004). According to the data of this study, wild fish consume 49.8, 40.0 and 47.3% POC, PON and TP, respectively, of the uneaten feed. To our knowledge, this is the first estimate of the effect of wild fish as a sink of exported POC, PON and TP in a commercial fish farm.

The consumption of food and faecal pellets by wild fish should help in the dispersion and mineralization of organic matter through digestion and physical transport before excretion (Vita et al., 2004). Wild fish aggregation seems to be a common event in aquaculture facilities around the world (Dempster et al., 2006) and, as this work shows, the phenomenon may be an important parameter when quantifying exported fish farm particulate organic wastes. To improve the forecasting capacity of particulate deposition models, estimates of the reduction the role of wild fish are necessary.

POC, PON and TP sedimentation rates showed an exponential decay and were fairly correlated with their respective concentrations in the sediment (Fig. 3). Nevertheless, TP appeared to be the best parameter for establishing the reach of fish farm particulate wastes, since it showed the highest correlation between sedimentation rate and sediment concentration. This agrees with other studies that found TP to be a sensitive indicator of aquaculture particulate waste products in the Mediterranean Sea (Karakassis et al., 1998; Holmer et al., 2007).

POC, PON and TP sedimentation rates were correlated with the chemical status of the sediment along the distance gradient studied, while their impact on the benthic community extended further. These findings suggest that the level of fish farm impact on the benthic community might be underestimated if it is assessed by only taking into account data (directly measured or from models) from particulate waste dispersion rates or measuring only physico-chemical parameters.

The macrofaunal community response to organic enrichment has been widely studied in many parts of the world and similar community patterns have been found (Pearson and Rosenberg, 1978). In this work, community parameters such as biomass and diversity did not follow the predicted pattern. Biomass did not increase with increased particulate organic input, and diversity did not peak at an intermediate impact level.

In the Mediterranean, the macrofaunal community response under the influence of marine aquaculture influence may not always follow the pattern described by Pearson and Rosenberg (1978), and may well show less marked increases of biomass (Karakassis et al., 2000; Apostolaki et al., 2007). This fact may be due to the carbonate sediments which predominate in the Mediterranean which are characterized by a lower iron content, in contrast with the terrigenous sediments typical of fjords and lochs where most studies have been performed. Iron can reduce the toxic effects of sulfides (the most important and toxic by-products of aquaculture organic matter) by binding to them and reducing their bioavailability to benthic organisms (Holmer et al., 2005). So, aquaculture-impacted sediments that contain iron would have fewer toxic effects, meaning that the local fauna could use the sources of food provided by aquaculture activities. Therefore, the carrying capacity of benthic communities related to organic matter input in the Mediterranean may be less than in temperate-cold regions where aquaculture normally develops in fjords and lochs.

Similarly, different communities may have different responses to impacts. Most of the studies made to date on benthic finfish farm impact have been performed on unvegetated beds. Very similar impact reach and levels have been found in Scotland, in Maërl beds at open sea sites (not enclosed, such us fjords and lochs) (Hall-Spencer et al., 2006). Consequently, the benthic impact assessment of aquaculture has to take into account that the chemical and biological changes produced may be highly dependent on parameters such as sediment type and the communities inhabiting the affected ecosystems.

Soft bottom ecosystems are fundamental for the functioning of marine systems (Olsgard et al., 2008), and the macrofauna inhabiting these ecosystems play an important role in benthic-pelagic coupling by enhancing microbial carbon oxidation and nutrient recycling (Kristensen, 2001). Nowadays, the preservation of diversity is a main concern and its relationship with ecosystem functioning is one of the major focuses of current ecology. In mesocosm experiments, it has been demonstrated that biodiversity is positively correlated with the ecosystem functioning, such us nutrient release (e.g. Emmerson et al., 2001).

On the other hand, there is an important information gap linking data from aquaculture particulate waste dispersion and community status. However, such knowledge is necessary for predicting the real level and reach of fish farming impact and for enabling protection agencies to adequately manage this activity. Cromey et al. (2002) created a waste dispersion model and established semi-empirical quantitative relationships between predicted solids accumulation and observed faunal benthic indices. Even so, it was difficult to describe a relationship for the suite of benthic indices except for the total individual abundance and the Infaunal Trophic Index (ITI), whose reliability for assessing community status is arguable (Maurer et al., 1999).

H' has proved to be amongst the best community status indicators (Giles, 2008). According to the diversity regression model obtained in this study, the increase in diversity starts to stabilize from around 200 m from the fish farm. The corresponding POC sedimentation rate at 200 m for the studied fish farm was 0.087 g m^{-2} day^{-1}. This value can be taken as the threshold for the community, above which diversity starts to be affected. This community is very sensitive to fish farming, and shows considerably low resistance (the threshold corresponds to an increase of 36 % of the basal POC input). As other work have shown, aquaculture is a major thread for Maërl species, mainly due to the increased oxygen demand in the ecosystem (Wilson et al., 2004).

In conclusion, the organic load exported from the fish farm studied, which has a highly efficient feeding system, is one magnitude order less than that observed in other fish farms reported in literature. Particulate deposition and dispersion models should include wild fish, since they may represent an important parameter when quantifying exported fish farm particulate wastes. Likewise, it has to be taken into account that the level of fish farm impact on the benthic community might be underestimated if it is assessed by only taking into account data (directly measured or from models) obtained from waste dispersion rates. Environmental agencies should define the maximum aquaculture waste loads by weighing up both economic interests and possible environmental impacts to each ecosystem type. Derived metrics and thresholds should be applied independently to each ecosystem according to its functions and unique characteristics.

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CHAPTER 4

The role of predation in the benthic system influenced by a fish farm with a moderate level of organic matter input

Abstract

To better understand the consequences of trophic structure alterations caused by different levels of pollution, trophic interactions need to be elucidated. This work shows that under the moderate organic enrichment conditions resulting from aquacultural activity, the effect of epibenthic predators on the seabed may be different to previously observed effects under high organic enrichment conditions. The level of organic matter enrichment may influence the type of epibenthic predators that will become predominant, and as a consequence, determine whether the ecosystem is more likely to show a three- or two-level trophic structure. Under low organic matter enrichment conditions, the main epibenthic predators are not demersal fish, but small invertebrate species, which seem to feed preferably on infaunal predators. Such feeding hardly produces any bioturbation activity and significantly decreases macrofaunal diversity. This decrease of diversity leads to a more reduced environment, which has important consequences for the biogeochemistry of the seabed. Our observations provide field evidence that strengthens the paradigm that considers diversity as a driver of ecosystem functioning.

INTRODUCTION

Organic matter enrichment is one of the main consequences of pollution due to human activity. Marine sediments are greatly influenced by the organic load received. Organic matter accumulation in the sediment leads to increases in oxygen consumption, contributing to the enhancement of anaerobic sediment metabolism, and the consequent production of its toxic by-products, mainly sulfides and ammonia (Brooks and Mahnken, 2003). This process results in profound chemical and biological changes in the seabed.

Many studies have thrown light on the dynamics of benthic communities along organic matter gradients (e.g. Pearson and Rosenberg, 1978; Hyland et al., 2005). However, predation effects have hardly been studied in this type of organic enriched ecosystems.

Aquaculture is one of the fastest growing human activities, which implies an increasing number of areas affected by organic input. In seabeds where fish farms are responsible for high levels of organic matter, predators, mainly demersal fish, can greatly reduce the benthic abundance of opportunistic species and organic matter accumulation, thus ameliorating organic enrichment on the seabed (Vita et al., 2004).

Despite so, aquaculture, is trying to become a more environmentally sound activity. Feeding is being optimized and there is a general trend to move fish farm facilities to more open and flushed locations (Henderson et al., 2001). Resulting in a decreased organic load to the benthic system, and only moderate organic enrichment conditions. Under these conditions, the inhabiting communities can better cope with the organic load, resulting in less pronounced changes in the sediment (Sanz-Lázaro et al. in prep).

Marine benthic systems have a high trophic complexity (Quijon and Snelgrove, 2008) and predation is one of the main factors responsible for this complexity. Predatory infauna have an important role in structuring benthic communities (Desroy et al., 1998) and so epibenthic predators, whether they feed preferentially on predatory infauna (three-level trophic structure hypothesis) or on the whole infauna (two-level trophic structure hypothesis) (Ambrose, 1984).

Despite this complexity, trophic interactions need to be better understood in order to elucidate the consequences of trophic structure alterations due to different pollution levels.

Demersal and motile epifauna, mainly fish, shrimps and crabs, feed on sediments (Danovaro et al., 2007). This activity may have a twofold effect: (1) predation of the infauna and (2) consumption of organic wastes, and sediment bioturbation while feeding.

Predation may produce changes in infaunal assemblages, densities and biomass or by consuming organic wastes and bioturbating the sediments, predators may increase mineralization rates (Katz et al., 2002). These last processes may lead to an increase in aerobic metabolism over the anaerobic, thus decreasing the accumulation of aquacultural wastes on the seabed and the toxic by-products of anaerobic metabolism.

Therefore, depending on which of the above effects prevails, very different responses will be observed in the infaunal communities and the biogeochemical cycles of the seabed.

However, the impact of predation in such moderate organic matter enrichment conditions has been little studied, and the studies that exist suggest that predation is taxon specific (Posey et al., 2006).

Although the use of cages in soft-sediment habitats is a probable cause for concern because of the potential for cage-induced habitat modifications, manipulative caging experiments have proved invaluable in extending our understanding of the complexity of local biological interactions in marine soft-sediments (Thrush, 1999; Beseres and Feller, 2007).

Focusing on the variation in the feeding rates of individual predators in response to external conditions can help in our understanding of the patterns of predation and so better predict the processes that make up benthic marine communities (Micheli, 1997).

To improve our knowledge of top-bottom effects in seabed affected by moderate organic matter enrichment and the consequences for sediment biogeochemical cycles, an experiment was carried out to disentangle the effects of each type of potential epibenthic predator (fish, crabs and shrimps) and of the predatory infauna on the benthic community. The aim, then was to test the predation effect on a benthic community under a low and constant organic matter enrichment regime due to aquaculture. We specifically wanted to test: 1) whether epibenthic predation has a significant effect in an ecosystem exposed to a low organic enrichment perturbation, and, if so, which types of epibenthic predator are the most influential, and 2) how predation affects the benthic community and the biogeochemistry of the seabed.

METHODS

Study area

The study was conducted below a fish cage at an open sea fish farm located in Águilas, SE Spain, (Western Mediterranean; 37º 24’ 56.2” N, 1º 32’ 4.0” W), which cultures guilthead sea bream (*Sparus aurata*) and sea-bass (*Dicentrarchus labrax*). The fish cage which was situated over the seabed where the study was performed, cultured 83,000 kg of guilthead sea bream. The mean deposition rate of the particulate organic carbon (POC) during the study period was 1.1 ± 0.4 g m^{-2} day^{-1}. The water temperature during sampling period ranged between 26 and 23 ºC at the surface and 19 and 16ºC at the bottom. The seabed depth was 31 m and the sediment was coarse to medium grade with a low percentage of silt and clay. The fish farm was located in a well flushed not unenclosed area.

The feeding system of the fish farm is very efficient since it uses of a floating silo, a computerized system and underwater cameras. The computerized system automatically distributes feed from the silo among the fish cages at an optimal rate and frequency, according to fish repletion, which is controlled by the fish farm staff through the use of underwater cameras. This leads to a considerable diminution of the organic matter load from the fish farm.

Experimental design

The effect of predation into the benthic system was studied by anchoring exclusion cages of different mesh size on the seabed below the above mentioned fish cage. All exclusion cages were made of plastic mesh (1 m x 1 m x 25 cm, length, width and height, respectively). In order to sequentially exclude predators we used different mesh sizes (2, 5, 10 and 30 mm) for the exclusion cages. The largest mesh size (30 mm) excluded most demersal fish); the 10 mm mesh size kept out big crabs and all fish; the 5 mm mesh size also excluded, most of the crabs and some shrimps; and the smallest mesh size (2 mm) excluded all the epibenthic predators, the predatory infauna the only predators.

Cage structures may result in artifacts, such as current disruption and modification of the deposition rates, which may be minimized by careful planning and cautious interpretation (Virnstein, 1979). In order to prevent undesired artifacts derived form current disruption, since different mesh sizes may have different effects, open-roof cages of the same mesh size were installed for each mesh size treatment. These open-roof cages were used as controls (from now on referred to as “controls”) to provide similar environmental conditions, but allowing predator access. Also, organic matter deposition rates could be altered by the mesh used to cover the upper part of the predation exclusion cages, which could either produce a decrease through a physical blocking effect, or an increase in the deposition rates, through a fouling effect of the inhabiting communities that colonize the mesh. In order to detect this type of artifact, particulate organic carbon (POC), particulate organic nitrogen (PON) and total phosphorous (TP) were measured in all the sediment samples at the end of the experiment.

The redox potential as well as ammonia were measured as the chemical sediment parameters used as indicators of the benthic status, since they have proved to be directly related with organic matter enrichment effects (Sanz-Lázaro and Marin, 2006; Giles, 2008). The faunal descriptors used to analyze the macrofaunal benthic community were abundance, biomass, species richness and Shannon Wienner diversity index (*H'*).

Before anchoring the cages to the seabed, it was checked that epibenthic predators were not included, while during the experiment, it was observed that open-roof cages did not attract fish or other predators. The exclusion cages were deployed for three months during the summer of 2006 before sediment samples were collected. In order to avoid edge effects, samples were always taken from the centre of the exclusion cage.

In order to minimize site variation and to ensure the most homogeneous conditions within replicates, all predation exclusion cages and controls were deployed (randomly) under the same fish cage.

Sediment sampling

To measure physico-chemical parameters, sediment samples were taken by divers using cylindrical hand-operated corers. Macrofaunal samples were taken using a hand grab (400 cm^2). The samples were immediately transported in containers to the laboratory.

Physico-chemical analysis

The organic matter content was measured by weight difference, heating dry sediment at 450 ºC for 5 hours. The redox potential was measured with an Orion ORP 91-80 electrode. Total ammonia nitrogen (TAN) of interstitial water was measured with an Orion 95-12 ammonia electrode. The sediment was first finely ground. Then, after a pre-treatment consisting of adding of HCl (1:1), particulate organic carbon (POC) and particulate organic nitrogen (PON) were determined using a Carlo Erba Inst. EA 1108 Elemental Analyser (Carlo Erba Strumentazione, Milan, Italy). Total phosphorus (TP) was determined following the 4500-PE Ascorbic acid method (APHA, 1995).

Biological analysis

Sediment samples were washed through a 1 mm sieve. The remaining sediment was fixed in a 4% formalin buffered solution, separated into major faunal groups and stored in a 70 % alcohol solution for later identification. Determination of benthic groups was made to the lowest possible taxonomic level. Species were grouped according to their feeding guilds following Pearson's (2001) and Fauchald and Jumars' (1979) recommendations. Macrofauna ash-free dry biomass was determined by weight difference after drying to constant weight at 60 ºC and subsequently burning at 450 ºC for 5 h.

Data treatment

A one-way ANOVA ($P < 0.05$) was performed between all the control treatments (open-roof cages) to test whether the different mesh sizes had a significant effect on the environmental variables, such as current alteration among others, which would lead to the misinterpretation of the predation effect on the measured parameters.

To test the effect of different predators on the measured parameters, a t-test ($P < 0.05$) was performed between each treatment and its control. The predation effect was analyzed pairwise separately for each treatment, in order to ensure that the effects of predation were not masked by the different mesh caging effect of each treatment.

If in these univariate tests, data did not meet normality or homocedasticity, a log(x+1) transformation was applied. If, after transformation the data still did not meet parametric assumptions, non-parametric tests were applied. The Mann-Whitney and Kruskal-Wallis tests ($P < 0.05$) were performed for pairwise and multiple sample comparisons, respectively.

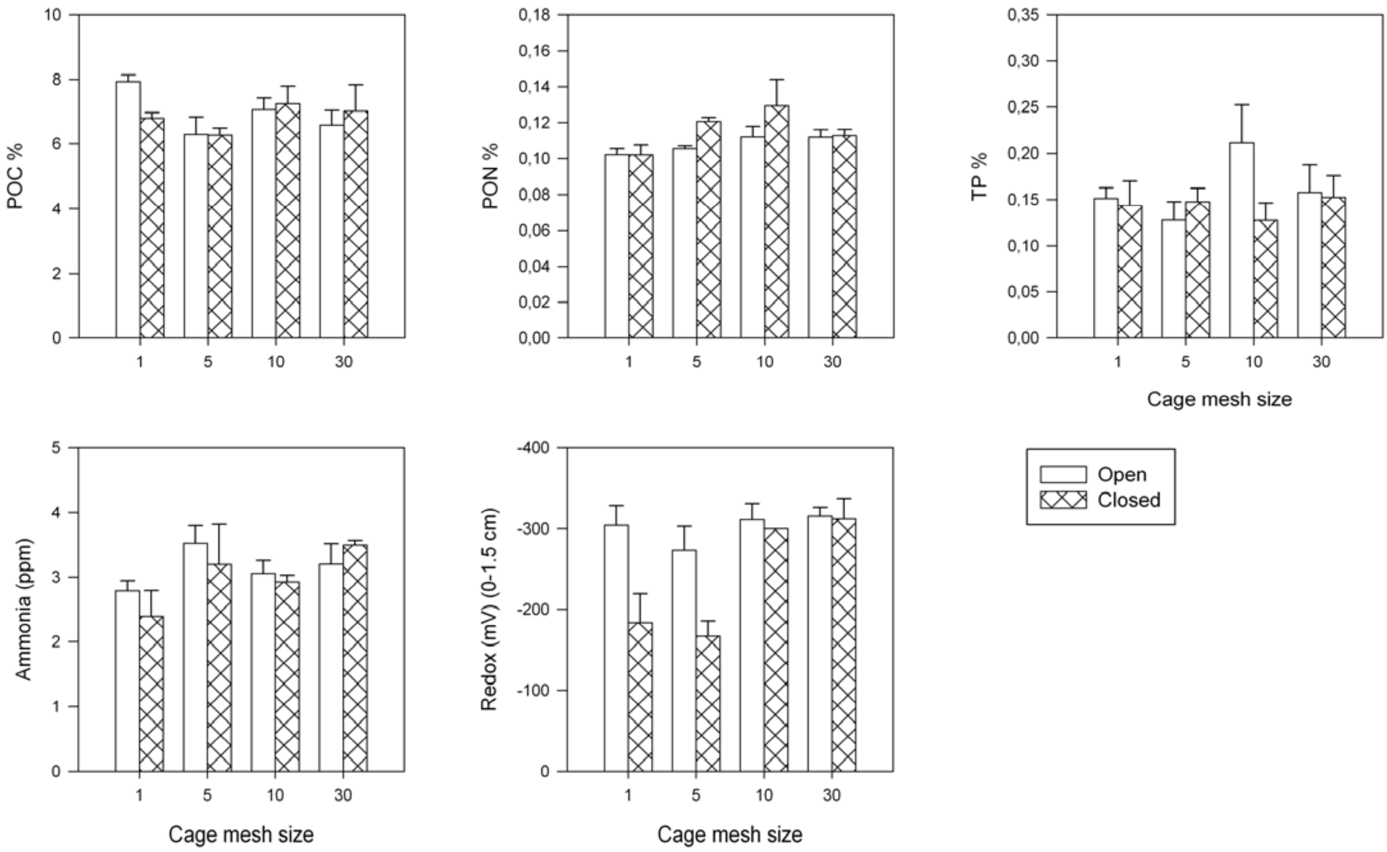
Multivariate analysis was applied to the macrofaunal abundance data using Primer (v6) software package. Non-parametric multidimensional scaling (MDS) ordination analysis (Clarke and Warwick, 1994) was performed to examine differences in the assemblages of taxa among treatments to represent the similarity between samples. A Bray–Curtis similarity matrix (Bray and Curtis, 1957) was calculated after a log (x+1) transformation. After MDS was plotted, a group-average clustering procedure based on Bray-Curtis similarity and SIMPROF was applied to find differences (significance level 5%) in the classification of the samples.

RESULTS

The benthic community showed moderate perturbation since *H'* was quite high (control values ranged between 2 and 3 bits) and species indicative of organic enrichment, such us capitellids, had a quite low abundance (mean density of the controls was below 190 indiv m^{-2}).

No significant differences were found between the control treatments for any of the measured parameters, indicating few undesired effects on the part of the cages used due to water current modification.

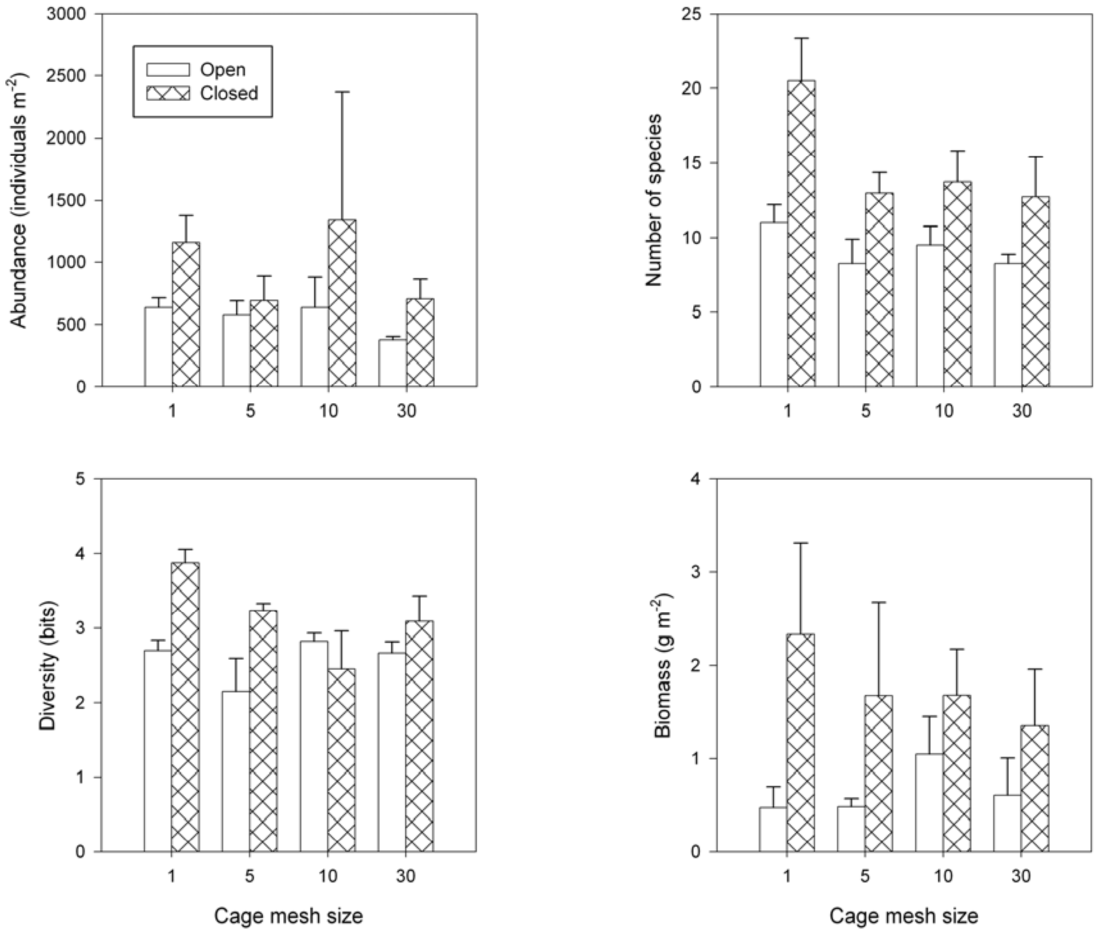
Fig 1. Sediment chemical parameters measured for the different treatments and respective controls (mean ± SE, n=4)



As regard POC, PON and TP, the values observed for each parameter were similar for most of the treatments. The POC concentration of the 2 mm mesh size treatment was significantly smaller than that of the control, even thought the POC concentration for that predation exclusion treatment was within the same range as the rest of the treatments. For PON, the concentration of the 5 mm mesh size treatment was significantly greater than in the control, but was also within the same range of the rest of the treatments. Thus, there was hardly any artifact effect caused by the exclusion cages used as regards the changes in organic matter deposition rates (Fig. 1).

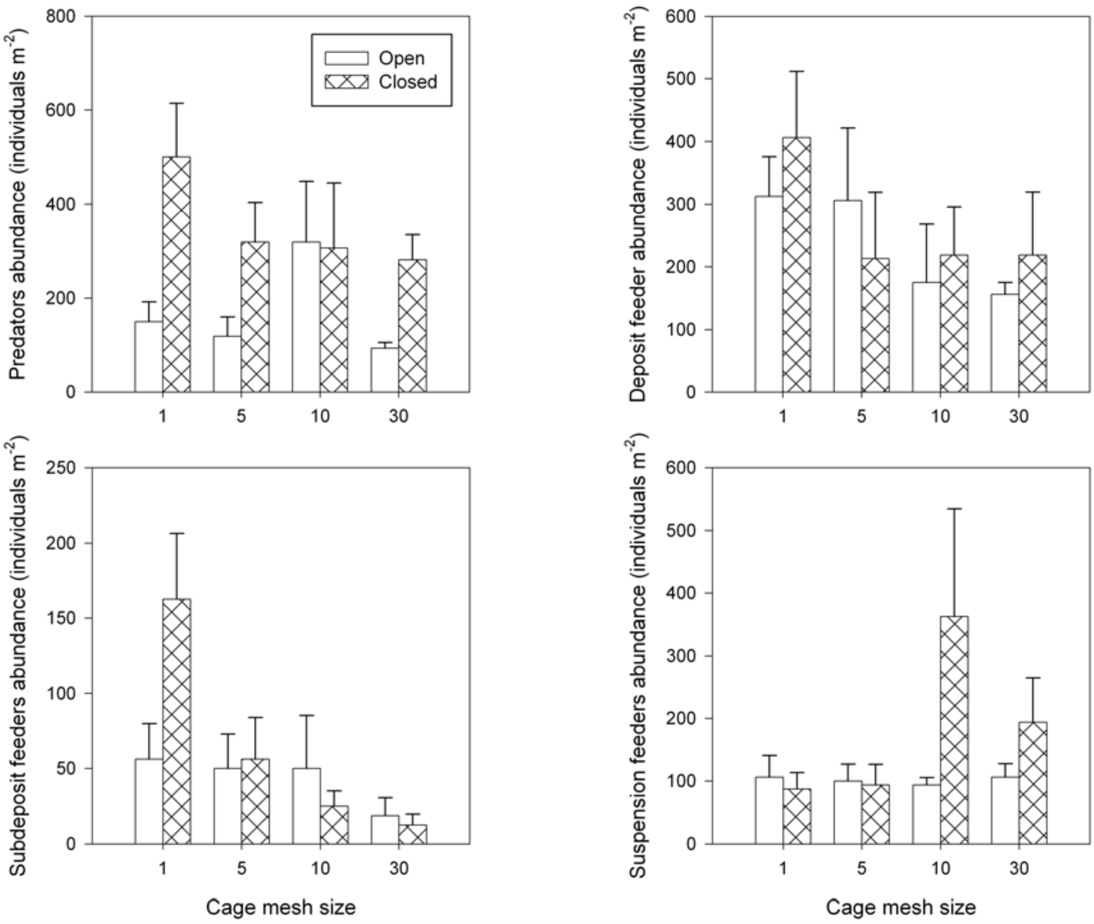
Redox values were significantly less reduced in the 2 and 5 mm treatments compared to their respective controls, and were much lower than in the rest of the treatments. Ammonia did not show significant differences for any treatment and the values were within the range of a low organic enrichment impact area (Sanz-Lázaro and Marin, 2006; Aguado-Gimenez et al., 2007).

Fig 2. Macrofaunal community descriptors values for the different treatments and respective controls (mean ± SE, n=4).



For community descriptors, abundance showed no significant differences for any treatment compared with its control, although the 2 mm mesh size treatment had a *P*-value of 0.060. Biomass values followed an inverse trend with the predator exclusion mesh size but had no significant differences either. Species richness and *H'* were only significantly different in the 2 mm mesh size treatment, although the 5 mm mesh size treatment was almost significant with a *P*-value of 0.068 and 0.053 for species richness and *H'*, respectively (Fig. 2).

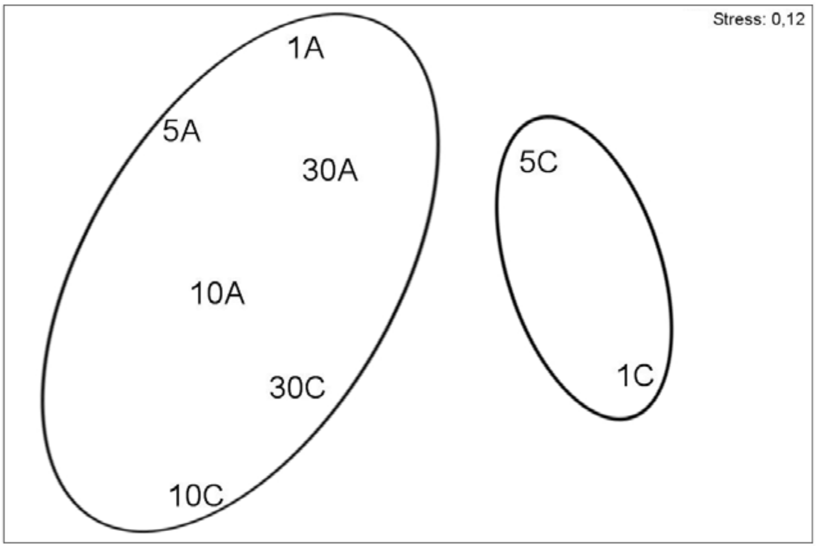
Fig 3. Macrofaunal density of the trophic groups for the different treatments and respective controls (mean ± SE, n=4).



The abundance of the predatory infaunal species was significantly different for the 2 and 30 mm mesh size treatments, although the 2 mm treatment was the only one that seemed to have a greater value compared with the rest of the treatments. The abundance of the rest of the trophic groups (deposit, sub-deposit and suspension feeders) did not show significant differences for any treatment, although subdeposit feeders also tended to increase with epibenthic predator removal (Fig 3).

MDS grouped, on one hand, the 2 and 5 mm mesh size treatments and, on the other hand, the rest of the treatments and all the controls. These two groups were significantly different according to SIMPROF analysis derived from the clustering classification analysis (Fig 4).

Fig 4. MDS based on species abundance. Groups are significantly different (significance level 5%) according to SIMPROF used in the cluster classification routine. For each sample, the number is the mesh size (mm) treatment, and the letter “A” indicates treatment (exclusion cage) while the letter “C” indicates the respective control (open exclusion cage). Each sample corresponds to the mean of each treatment or control (n=4).



DISCUSSION

In the present study, exclusion cages allowed us to test for the cumulative impact of different size epibenthic predators and of predatory infaunal species in a subtidal marine sedimentary habitat under moderate organic enrichment conditions. The use of exclusion cages of different mesh size allowed us to differentiate the extent and effect of different predators on the benthic system.

Undesired caging artifacts/effects were tested in order to see if they were relevant and could mask the predation effect. Different mesh sizes could have two kinds of caging effect: (1) lateral effects, which would mainly reduce the water current and in turn have important consequences such as decreasing dissolved oxygen in the water layer close to the sediment surface, and (2) upper effects, which may reduce the organic matter deposition rates due to the mesh retention effect, or increase the deposition rate as a result of the fouling effect of the epiphitic communities that can colonize the mesh.

Lateral undesired effects due to caging artifacts did not seem to be important, since the one-way ANOVA between all the controls for all the measured parameters did not show significant differences, indicating the low effect of the cages used on particle accumulation due to a hypothetical reduction on the water current intensity. Redox values in the first 1.5 cm of the sediment were within a small range for the different control treatments and values did not show any trend in response to increasing/decreasing mesh size. Thus, all this indicates that the different mesh sizes ad little effect on the reduction of water current intensity and on the oxygen concentration in the water column close to the sediment.

As regards upper undesired effects, deposition rates of organic matter to the sediment did not seem to be modified. POC, PON and TP values were similar for all predation exclusion treatments. Possible shading effects as regards benthic microalgae growth did not seem to be important compared with the enormous shading effect of the fish cage placed over the seabed of the experiment area. Therefore, it can be assumed that the effects derived from the results of this study are mainly due to predation.

Marine benthic systems have a high trophic complexity and predation is one of the main factors responsible for this complexity. In these systems trophic chains are not as clearly defined as in some terrestrial and water column systems and many infaunal species possess facultative trophic habits (Hiddink et al., 2002).

There is much controversy about whether marine sedimentary communities have a three- or two-level trophic structure. The main factor that differentiates between the two is whether the epibenthic fauna feed preferentially or not on the predators of the infauna, respectively (Quijon and Snelgrove, 2008).

In this work, the main epibenthic predators seen to regulate the trophic structure are not fish but smaller invertebrates such as small crabs and shrimps. These species do not seem to have the same type of feeding as the above mentioned fish. They hardly bioturbate the sediment while feeding (Beseres and Feller, 2007), since no increases in oxidation in the sediment due to this activity were observed in this experiment. These epibenthic predators seem to be more specific, mainly feeding on predators, but also on sub-deposit feeders to some extent.

Under organic enrichment conditions, when the organic load is high, the epibenthic predators that seem to mainly regulate the macrofaunal community structure are demersal fish species (Vita et al., 2004). These species show a mullet-type feeding by ploughing the sediment, which is very unspecific (Marin pers obs). In the study carried out by Vita et al. (2004), these fish fed mainly on non-predatory infauna, capitellids, which are by far the dominant taxa in such heavily impacted sediments. These fish, through this type of feeding, bioturbate the sediments, decreasing to some extent the reduced conditions due to the effects of organic enrichment (Katz et al., 2002).

Therefore, the feeding specifity of the epibenthic fauna may influence a community to behave more like a three- or two-level trophic structure. In turn, the epibenthic fauna can be highly influenced by sediment characteristics (Thrush, 1999 and cites there in) such as the degree/level of pollution (in our case, organic enrichment). In this type of ecosystems, where trophic relations possess a high degree of complexity, it may be too simplistic to consider them as having solely a three- or two-level trophic structure. These ecosystems may act as one or another type, depending on several factors and also could act as a mixture of both types, having epibenthic predators that feed preferentially on some infauna taxa but not necessarily on predatory infauna (Posey et al., 2006).

In this work, predatory infaunal species significantly increase in abundance when all epibenthic predators are excluded, thus regulating macrofaunal assemblages. Even though, predation by epibenthic and predatory infaunal species has very differing effects.

Compared with epibenthic predators, the predation performed by predatory infaunal species, seem to be less intense, and produce significantly different community assemblages with higher diversity. In turn, these assemblages of higher diversity seem to improve the benthic status of this moderately impacted community, creating a less reduced environment in the upper layers of the sediment. This, enhances aerobic metabolism, which has more efficient mineralization rates, and, in turn, decreases organic matter accumulation, diminishing anaerobic metabolism and its derived toxic by-products.

Furthermore, predation seems to have different effects on the benthic community according to the specific disturbance level scenarios of each location. In very impacted ecosystems such as in Vita et al. (2004) where the carbon deposition rates are high and benthic communities may not cope with the organic matter accumulation, demersal fish may help to lessen organic matter accumulation by increasing mineralization rates. This may be done directly by consuming deposited organic matter, or indirectly, by bioturbating the sediments, creating a more favourable environment for bacteria with aerobic metabolism and with higher carbon mineralization rates. Demersal fish, apart from consuming organic matter, may also have an important predation effect on the macrofauna. Impacted communities have a low number of opportunistic species, which are very abundant. The demersal fish would mainly reduce the density of these opportunists. These types of species, such as capitellids, usually have small bodies with low bioirrigation capacity. Therefore, in this scenario, predation by demersal fish has little effect on macrofaunal diversity and on the macrofaunal-enhancing effect of increasing mineralization rates, which is less influential than the organic matter consumption and bioirrigation produced by demersal fish.

However, in areas where the organic matter load is moderate enough for the benthic community to cope with it to some extent, sediment assemblages show slight changes, maintaining a considerable high diversity and larger infaunal organisms (Pearson and Rosenberg, 1978). Large infaunal organisms are more likely to possess bioirrigation activity- and different species may have different bioirrigation traits. The feeding strategies of epibenthic fauna under low organic enrichment conditions do not seem to involve a high bioturbation activity, compared with the above mentioned effect of demersal fish, and their predation activity is more taxon-specific. In this way, they reduce diversity and, consequently decrease bioirrigation activity in the sediment, leaving the benthic system with fewer bioturbating-type species which pump water into the sediment in an attempt to cope with the oxygen debt produced by the organic load (Heilskov et al., 2006).

Nowadays, there is paradigm shift which considers biodiversity not only as a consequence, but also as an important driver of ecosystem properties and processes (Gamfeldt and Hillebrand, 2008). Most of the experiments performed related with this issue are mesocosm studies, and share the common criticism, they lack the complexity of natural ecosystems, using artificially assembled communities with random species loss (Loreau and Hector, 2001). This study provides field evidence for this new paradigm. In a naturally assembled community, predation produces a loss of diversity, which leads to a decrease in ecosystem efficiency to cope with the stress due to organic matter enrichment conditions.

This study, along with Vita et al. (2004) and Sanz-Lázaro et al. (in prep), helps elucidate the role of wild fish in the benthic system influenced by marine finfish farms. Pelagic wild fish seem to have a positive indirect effect on the benthic system by reducing the organic load that reaches the seabed (Vita et al., 2004; Felsing et al., 2005; Sanz-Lázaro et al. in prep). Under a high organic matter enrichment scenario (due to poor husbandry practices and enclosed fish farm locations), demersal wild fish have a positive effect on the severely impacted seabed. Wild fish predation seems to be non-specific, and so there is a two-level trophic structure system.

With lower organic matter loads, as a result of improvements in feeding efficiency, and sitting aquaculture facilities in well flushed and open sea areas, wild demersal fish do not seem to play an important role on seabed predation. In this situation, benthic ecosystems seem to be top-down controlled by small epibenthic invertebrates. These species seem to have a negative effect on the benthic status, worsening the effects of the organic matter enrichment conditions, by decreasing macrofaunal diversity and, in turn, creating a more reduced environment. In this case, epibenthic invertebrate predators seem to preferentially predate on predatory infauna, leading the system to have a more three-level type trophic structure.

In conclusion, this work helps elucidate how trophic control works in marine sedimentary habitats under organic matter enrichment conditions. Marine sedimentary communities can act like three- or two-level trophic structures, according to the type of epibenthic predators, which, in turn, can be determined by other factors, such as the perturbation level of the ecosystem. Under low organic enrichment conditions, epibenthic predators seem to diminish macrofaunal diversity, which causes a more reduced environment, with important biogeochemical consequences. The study also provides field evidence that strengthens the paradigm that considers diversity as a driver of ecosystem functioning.

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CHAPTER 5

Benthic recovery during open sea fish farming abatement in Western Mediterranean, Spain

Abstract

Fish farming is an important source of organic matter input in coastal waters, which contributes to eutrophication. In this study, the macrofaunal benthic community was studied after the cessation of fish farming with the aim of improving our understanding of benthic succession and sediment recovery in a marine ecosystem. The results showed that the best environmental variables for assessing organic pollution were acid-volatile sulfides (AVS) and redox potential. Succession and recovery was best explained by macrofaunal analysis based on community composition as well as on trophic groups. The patterns of recovery differed between each impacted station. For this reason, succession could not be accurately predicted due to the unique environmental parameters and the singular community functional structure of each location. The Azti Marine Benthic Index (AMBI) proved its validity for assessing pollution but did not distinguish between successional stages.

INTRODUCTION

During recent decades, fish farming in the open sea has undergone almost exponential growth (FAO, 2004). Fish-farms produce a large quantity of wastes (Gowen and Bradbury, 1987), which results in the accumulation of organic matter on bottom sediments, causing severe modifications of the physical and chemical characteristics of the benthic environment (Diaz and Rosenberg, 1995; Karakassis et al., 2000). Many studies have focused on processes related to the environmental impact produced by aquaculture, using macrofaunal analysis and measuring a great number of environmental variables (Karakassis et al., 2000; Pawar et al., 2002). But very few studies have focused on the benthic recovery after fish farming cessation.

In previous studies of benthic recovery after fish farming cessation (Karakassis et al., 1999; Brooks et al., 2003; Pereira et al., 2004), the recovery rates observed in the different experiments differed to a large extent. In Greece (Karakassis et al., 1999), total benthic recovery had not been achieved after 23 months, while in British Columbia, Brooks et al. (2003) reported complete biological remediation after 6 months. At a Scottish sea loch, Pereira et al. (2004) found that sampled stations were highly to moderately disturbed after 15 months. In all these experiments, recovery was considered to have been achieved when benthic fauna assemblages were similar to those of control stations.

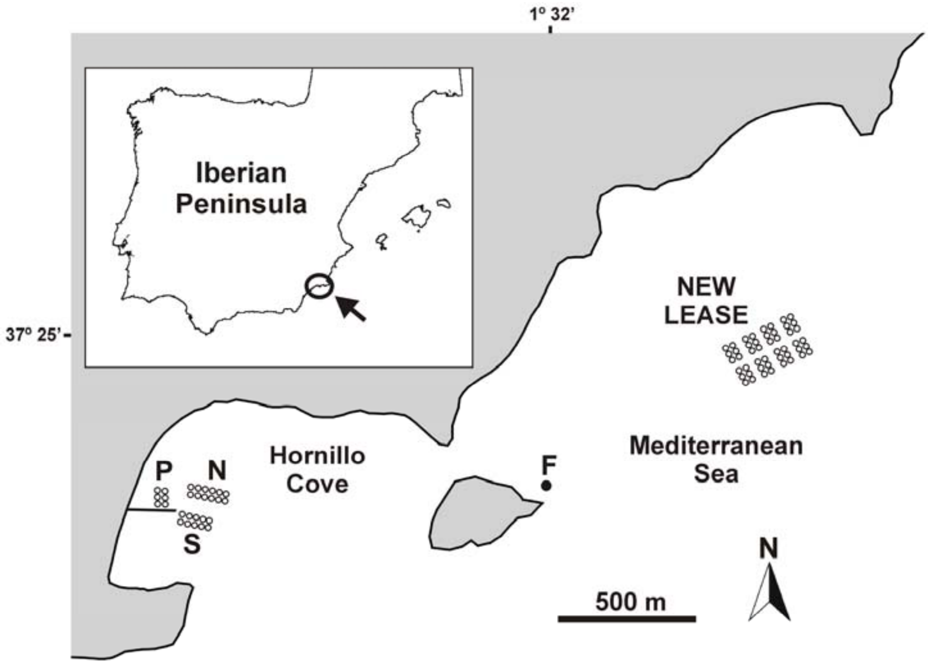
The study was carried out at a fish farm located in the Mediterranean Sea on the SE coast of Spain. At the time of the study, fish culture had been practised for more than a decade, with a mean fish biomass of between 30 and 60 ton per year. From January 2001 to March 2003, the installation was progressively dismantled and fish were transferred to another farm located 3 km NE. The singularity and interest of this study is based on two facts: (1) fish culture abatement involved different groups of cages at different times, which enabled us to study, the way succession occurs before, during and after organic pollution abatement in different locations within a single site over 2 years; (2) for a period of two months, in the summer of 2002, production increased enormously as extra fish cages were deployed. This fact produced substantial disturbance in the surroundings, including the sampled stations, each of which was in a different stage of succession at the time of the disturbance. The aim of the study was to monitor the three different groups of fish cages of the same fish-farm, which were in different stages of succession.

METHODS

Location and sampling

The study area was located at Hornillo Cove, Águilas, SE Spain (Western Mediterranean) (Fig. 1). The cove has an area of approximately 700,000 m^2 with an average depth of 21 m and a maximum depth of 37 m.

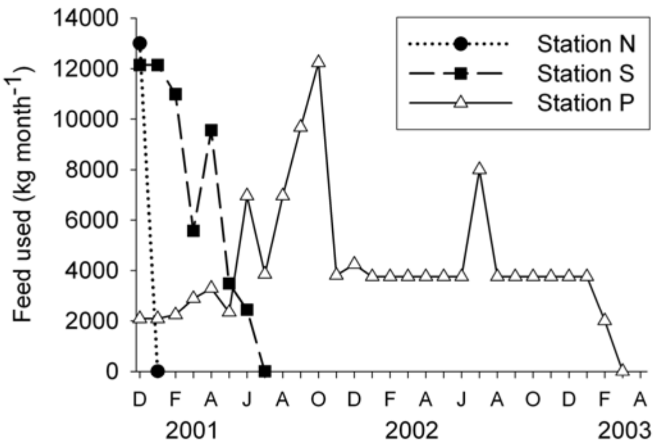
Figure 1. Location of Hornillo Bay and sampling sites: N (37º 24’ 34.5” N, 1º 33’ 26.6” W), S (37º 24’ 30.2” N, 1º 33’ 28.9” W), P (37º 24’ 32.3” N, 1º 33’ 33.1” W) and F, (37º 24’ 33.3” N, 1º 32’ 35.9” W)



Four stations were sampled. Replicates were taken over an area of 10 m from the anchoring point. Three stations (N, S, and P) corresponded to each of the different fish cage groups (Fig. 1). The reference station, F, was chosen outside the cove due to its biotic and physico-chemical resemblance with the other stations. The depth for each station was N: 14 m, S: 18 m, P: 15 m and F: 20 m.

The fish farm had produced guilthead sea bream (*Sparus aurata*) and sea-bass (*Dicentrarchus labrax*) since 1989. During the last year of full production (2000) the cultured fish biomass was around 12, 12 and 6 ton and feeding rate was 13, 12 and 2 metric ton of food per month for stations N, S and P, respectively. During abatement, the cultured fish biomass and feeding rate fluctuated (Fig. 2). The cages were removed in January 2001, July 2001 and March 2003 (N, S and P stations, respectively), and moved to the new area leased for fish farming.

Figure 2. Feeding rate of the different fish cage groups from December 2000 until April 2003. Data of station P from January 2002 until April 2003 was estimated according to the number of fish cultured due to the inexistence of feeding rate data for this period of time



Sampling was carried out in October 2001, January 2002, May 2002, October 2002, April 2003, July 2003 and November 2003. In the first survey, only stations N, S and P were sampled, while all four stations were sampled in subsequent surveys. Four replicate samples were taken from each station at every sampling time. All the samples were collected by scuba divers.

Physico-chemical analysis

Physico-chemical sediment parameters such as redox potential, ammonia, acid-volatile sulfides (AVS), organic matter, carbon–nitrogen ratio (C:N) and grain size were measured. For the granulometric analysis, sediment samples were first dried at 60 ºC and then sieved through a series of sieves on a mechanical sieve shaker (Buchanan, 1984). Redox potential was measured in sediment cores of 6 cm diameter and 25 cm length, which were immediately frozen. In the laboratory, cores were thawed, sliced into 2 cm sections and redox potential was measured with an Orion ORP 91-80 electrode. To measure ammonia, interstitial water was extracted from the top 2–3 cm of the sediment. The ammonia content was measured with an Orion 95-12 ammonia electrode. The sediment samples used for measuring sulfides were stored in plastic bags without air bubbles to prevent oxidation and were frozen until analysis. The sulfide content was extracted and measured following Allen's protocol (Allen et al., 1993). The organic matter content was measured by weight difference, heating dry sediment at 450 ºC for 5 h. The atomic C/N ratio was measured by Elemental Analyzer C, N model EA1108 by a Carlo Erba Instrument. Total organic carbon was also measured using the same equipment after a treatment with 2 N HCl and then drying at 105 ºC.

A two-way factorial analysis of variance (ANOVA) was used for testing significant differences between stations and times for all the physico-chemical sediment parameters. ANOVA was performed after checking for normality with Kolmogorov–Smirnov's test and homogeneity of variances with Levene's test. To achieve normality, values were log (x+1) transformed.

Biological analysis

Macrofaunal samples were taken using a hand grab (400 cm^2). Samples were washed through a 0.5 mm sieve. The remaining sediment was fixed in a 4% formalin buffered solution, separated into major faunal groups and stored in a 70% alcohol solution for later identification. Determination of benthic groups was made to the lowest possible taxonomic level.

Using macrofaunal data, abundance and species richness were obtained. These parameters were used to calculate the faunal descriptive Shannon–Wiener index for measuring diversity.

The Azti Marine Biotic Index (AMBI) was calculated using the September 2004 species list. This index assesses environmental benthic quality by quantifying the occurrence of macrofaunal species. The species are divided into five groups according to their sensitivity to an increasing stress, obtaining a marine biotic index (BI). Because of the limitations of using such an index with discrete values, the continuous index, biotic coefficient (BC) was obtained (Borja et al., 2000). AMBI was applied to see how well it assessed organic enrichment and to compare it with the other tools used.

Taxa that contributed <4% to the total abundance were removed from the dataset and a Bray–Curtis similarity matrix (Bray and Curtis, 1957) was calculated after transformation of log (x+1). Non-parametric multidimensional scaling (MDS) ordination analysis (Clarke and Warwick, 1994) was performed to represent the similarity between the samples (MDS-A). The BioEnv routine was used to find which environmental parameters best explained the MDS pattern, based on the macrofaunal data. Percentage of organic matter, percentage of silt and clay (fines), ammonia, AVS, redox potential (measured between 0 and 2 cm at the sediment surface) and the C/N ratio were used as environmental variables. BioEnv was performed using Spearman's rank correlation. The SIMPER routine was applied to know which species were the most important in terms of their contribution to the similarity or dissimilarity and to identify indicator species in different stages of succession. Analysis was made with a cut-off of 90%. Multivariate analyses were obtained with the statistical package Primer version 5.

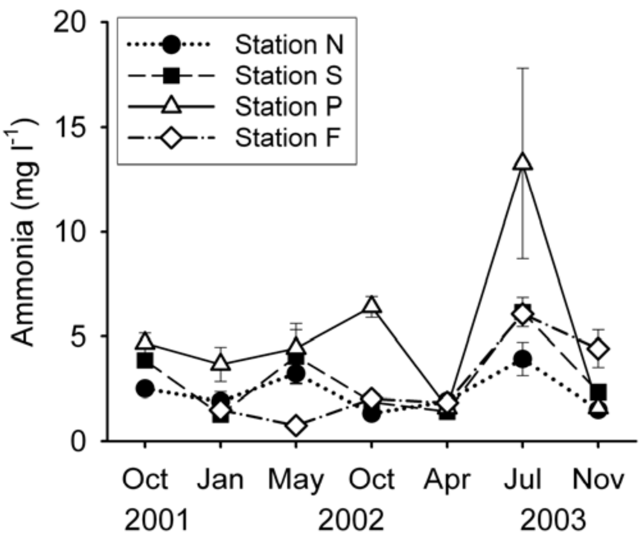
Functionality was compared between the different stations and periods of time by grouping species according to their feeding guilds following Pearson's (2001) recommendations without considering absorbers due to our lack of knowledge of species with such a feeding habit. The trophic groups included: predators, surface deposit feeders, sub-surface deposit feeders, suspension feeders and grazers. Taxa were associated to their feeding guild according to bibliography as well as our own previous knowledge. Feeding guild abundance was plotted against time, as well as by MDS (MDS-F). The MDS was also based on a Bray–Curtis similarity matrix with a transformation of log (x+1).

RESULTS

Physico-chemical parameters

Organic matter did not show any marked trend during the studied period. The silt and clay percentage remained stable for all the stations, except N, where it decreased (Table 1). Ammonia values showed similar levels in stations N, S and F but where higher in P most of the time (Fig. 3). AVS values remained low in the reference station throughout the experiment compared with the impacted stations, where the values clearly fell, although at different rates (Fig. 4).

Figure 3. Temporal changes in ammonia concentration in the interstitial water extracted from the top 2-3 cm of sediment (mean ± SE, n=4)



The redox potential depth was fairly stable at the reference station (F) and only towards the end of the surveyed period did it slightly decrease. In all the other stations, to a greater or lesser extent, redox potential showed a rising trend. At the last survey time all the redox values for the top 2 cm were positive or only slightly negative. Station N showed the lowest redox values (Fig. 5).

Figure 4. Temporal fluctuations in acid volatile sulphides (AVS) in the top 2-3 cm of sediment (mean ± SE, n=4)

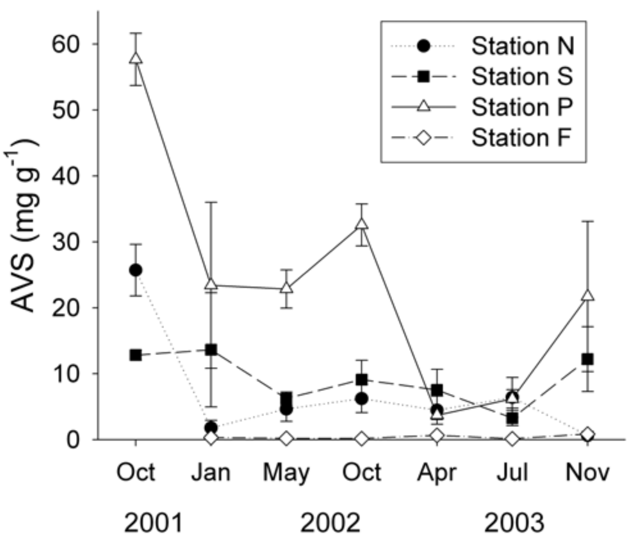
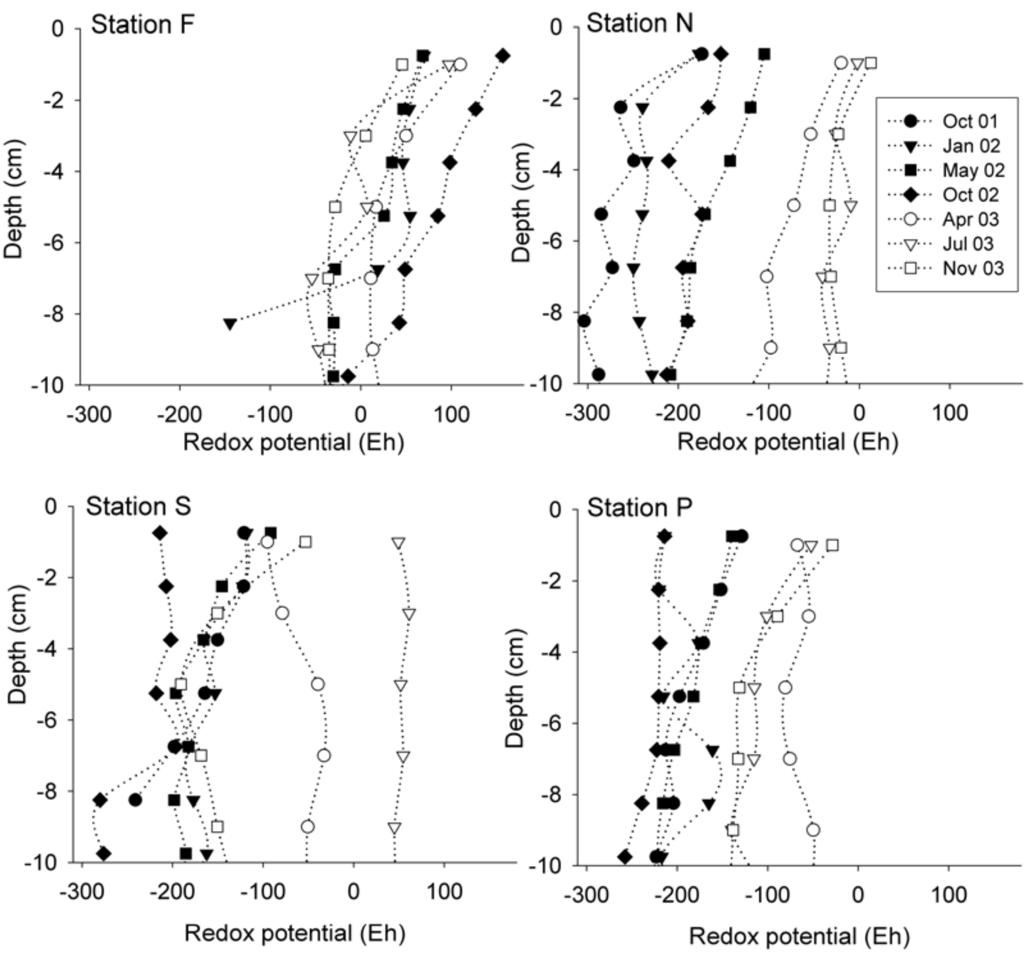


Figure 5. Temporal variation in REDOX profiles in the top 10 cm of sediment (mean, n=4)



The atomic C/N ratio at station P presented the lowest values while the reference station showed the highest values for the most of the surveyed time. Organically enriched stations (N, S and P) showed an initial rise and then a fall to the initial levels (Table 1).

Table 1. Organic matter, % silt / clay (i.e. < 63 μm) and C:N ratio data (mean ± SD, n=4). For location of sampling stations F, N, S and P, see Fig. 1.

Date	Station	% Organic matter	% silt / clay	C:N
Oct 01	N	4.3 ± 1.29	14.5 ± 6.19	9.1 ± 0
	S	2.6 ± 0.38	5.5 ± 1.85	9.6 ± 0
	P	2.8 ± 1.16	3.4 ± 0.95	6.3 ± 0.53
Jan 02	F	1.9 ± 0.29	1.2 ± 0.15	19.4 ± 9.16
	N	8.3 ± 2.8	11.0 ± 2.18	12.4 ± 1.67
	S	4.3 ± 2.38	3.6 ± 0.57	24.0 ± 19.55
	P	6.4 ± 3.49	3.6 ± 0.58	10.4 ± 2.40
May 02	F	1.8 ± 0.13	1.5 ± 0.22	13.3 ± 3.45
	N	14.0 ± 1.98	14.7 ± 3.63	16.2 ± 5.06
	S	6.5 ± 3.07	5.5 ± 1.04	17.1 ± 4.48
	P	3.9 ± 0.31	4.5 ± 0.70	11.1 ± 2.48
Oct 02	F	1.6 ± 0.11	1.0 ± 0.48	13.9 ± 3.67
	N	5.3 ± 1.56	13.6 ± 6.10	8.6 ± 1.62
	S	3.4 ± 0.85	3.3 ± 0.82	7.5 ± 1.77
	P	2.1 ± 0.35	2.8 ± 0.53	13.0 ± 1.82
Apr 03	F	4.1 ± 0.33	2.9 ± 0.73	6.3 ± 1.23
	N	3.8 ± 0.50	5.5 ± 0.77	6.6 ± 1.31
	S	3.7 ± 1.02	3.7 ± 0.80	14.4 ± 5.87
	P	1.6 ± 0.56	1.5 ± 0.53	6.2 ± 1.70
Jul 03	F	3.9 ± 0.34	3.8 ± 0.97	7.8 ± 1.24
	N	4.0 ± 0.53	5.9 ± 2.49	6.3 ± 1.29
	S	3.5 ± 0.34	2.0 ± 0.22	4.2 ± 0.23
	P	3.2 ± 1.07	1.9 ± 0.53	3.7 ± 0.77
Nov 03	F	4.1 ± 0.47	4.0 ± 1.07	9.0 ± 0.67
	N	3.6 ± 0.42	3.8 ± 0.75	7.8 ± 1.42
	S	4.2 ± 0.25	4.4 ± 0.91	6.0 ± 0.44
	P	2.9 ± 0.21	1.6 ± 0.30	8.4 ± 4.80

ANOVA pointed to significant temporal and spatial differences ($P < 0.001$) for the physico-chemical sediment parameters analyzed (Table 2). These results showed that the surveyed sediment parameters varied greatly in time as in space due to changes in organic input.

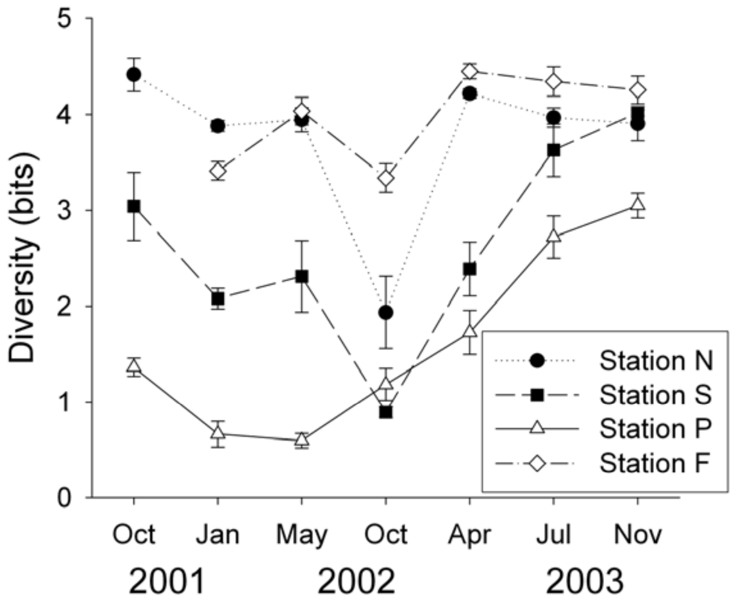
Table 2. Two-way factorial ANOVA without replication on differences in physico-chemical sediment parameters among the effects, stations (df=3) and times (df=6).

Variable	Source of variability	F	p
AVS	Station	107.748	<0.001
	Time	9.975	<0.001
Ammonia	Station	21.886	<0.001
	Time	34.165	<0.001
Redox (-2 cm)	Station	49.436	<0.001
	Time	16.757	<0.001
% organic matter	Station	51.944	<0.001
	Time	13.230	<0.001
% silt / clay	Station	176.353	<0.001
	Time	10.376	<0.001
C:N	Station	12.645	<0.001
	Time	28.564	<0.001

Biological analysis

A total of 17,637 individuals corresponding to 184 taxa were collected. The organically enriched stations showed similar behaviour as far as the Shannon–Wiener is concerned, decreasing at first and ending with a marked increase, with values similar to the reference station (Fig. 6). The values recorded at the reference station remained fairly constant. The Shannon–Wiener index for stations N and S clearly decreased in the October 2002 survey.

Figure 6. Changes in Shannon-Wiener diversity index (mean ± SE, n=4).



According to the AMBI pollution classification (Table 3), the reference (F) and station N were unpolluted, except in October 2002. Station S was slightly polluted except in January 2002 and November 2003. The last two surveys classified station P as unpolluted.

Feeding guild abundance fluctuated in N, S and P, especially as regards the surface deposit feeder group (Fig. 7). Stations N and S showed the highest variation in the October 2002 survey, while at station P the most important peaks of surface deposit feeder abundance were observed in May 2002 and April 2003. At stations N, S and P the ratio between the different feeding guilds underwent substantial variations. At reference station (F), this ratio was more stable, only presenting a slight peak (corresponding to the predator group) in April and July 2003.

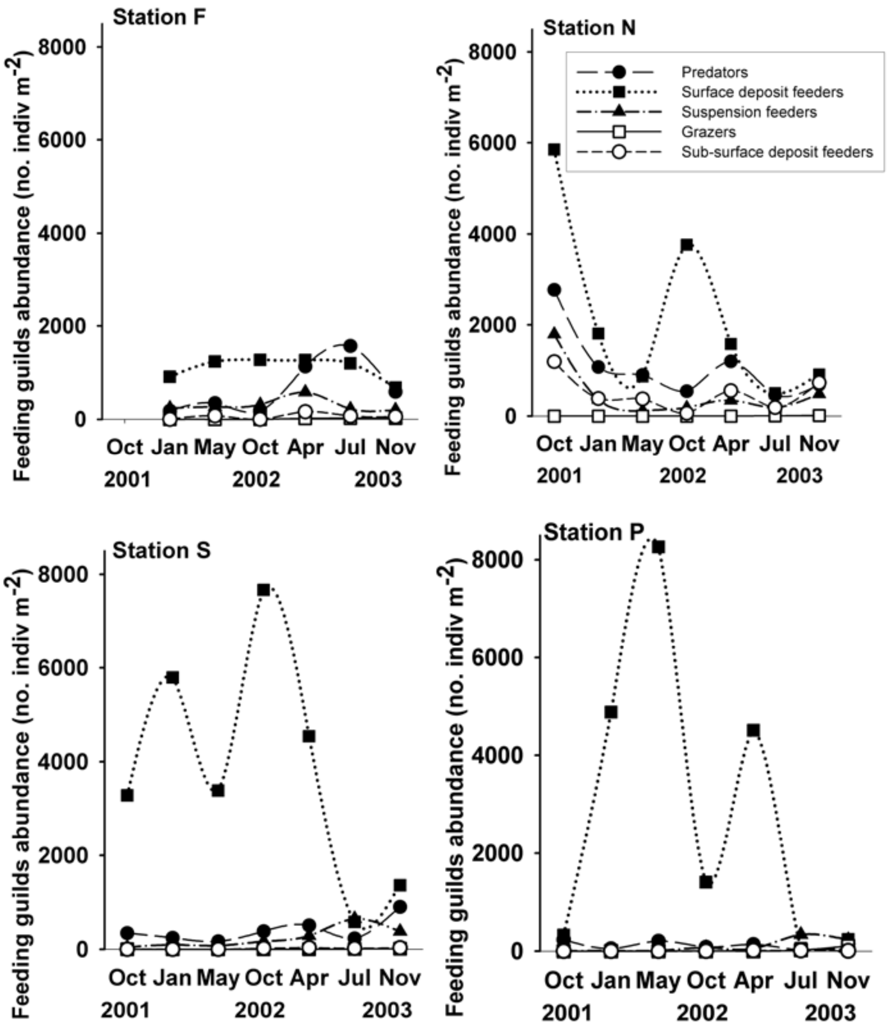
MDS based on species abundance (MDS-A) as well as MDS based on feeding guild abundance (MDS-F) indicated a clear trend for station N, followed by station S, to approximate the reference station. Station P showed a much lower degree of similarity with the rest of the stations, approximating the reference station much more slowly and fluctuating greatly in position (Fig. 8a and b). First surveys of station S (SA, SB and SC) share considerable similarities with mid-time surveys of station P (PB, PC, PD and PE). Station P during July and November 2003 surveys showed very low similarity to other stations. The difference was mainly due to the lack of some functional groups at station P during most of the surveys and at station S during the first surveys.

Table 3. AMBI biotic coefficient (BC), biotic index (BI) and pollution classification. For location of sampling stations F, N, S and P, see Fig. 1.

Date	Station	BC	BI	Pollution Classification
Oct 01	N	0.694	1	Unpolluted
	S	1.882	2	Slightly polluted
	P	4.274	3	Moderately polluted
Jan 02	F	0.363	1	Unpolluted
	N	0.717	1	Unpolluted
	S	3.971	3	Moderately polluted
	P	5.582	6	Heavily polluted
May 02	F	1.087	1	Unpolluted
	N	1.057	1	Unpolluted
	S	3.156	2	Slightly polluted
	P	5.666	6	Heavily polluted
Oct 02	F	1.367	2	Slightly polluted
	N	2.436	2	Slightly polluted
	S	2.815	2	Slightly polluted
	P	5.323	5	Heavily polluted
Apr 03	F	1.106	1	Unpolluted
	N	1.015	1	Unpolluted
	S	2.305	2	Slightly polluted
	P	4.235	3	Moderately polluted
Jul 03	F	1.107	1	Unpolluted
	N	1.063	1	Unpolluted
	S	1.458	2	Slightly polluted
	P	0.138	0	Unpolluted
Nov 03	F	0.730	1	Unpolluted
	N	0.476	1	Unpolluted
	S	1.018	1	Unpolluted
	P	0.469	1	Unpolluted

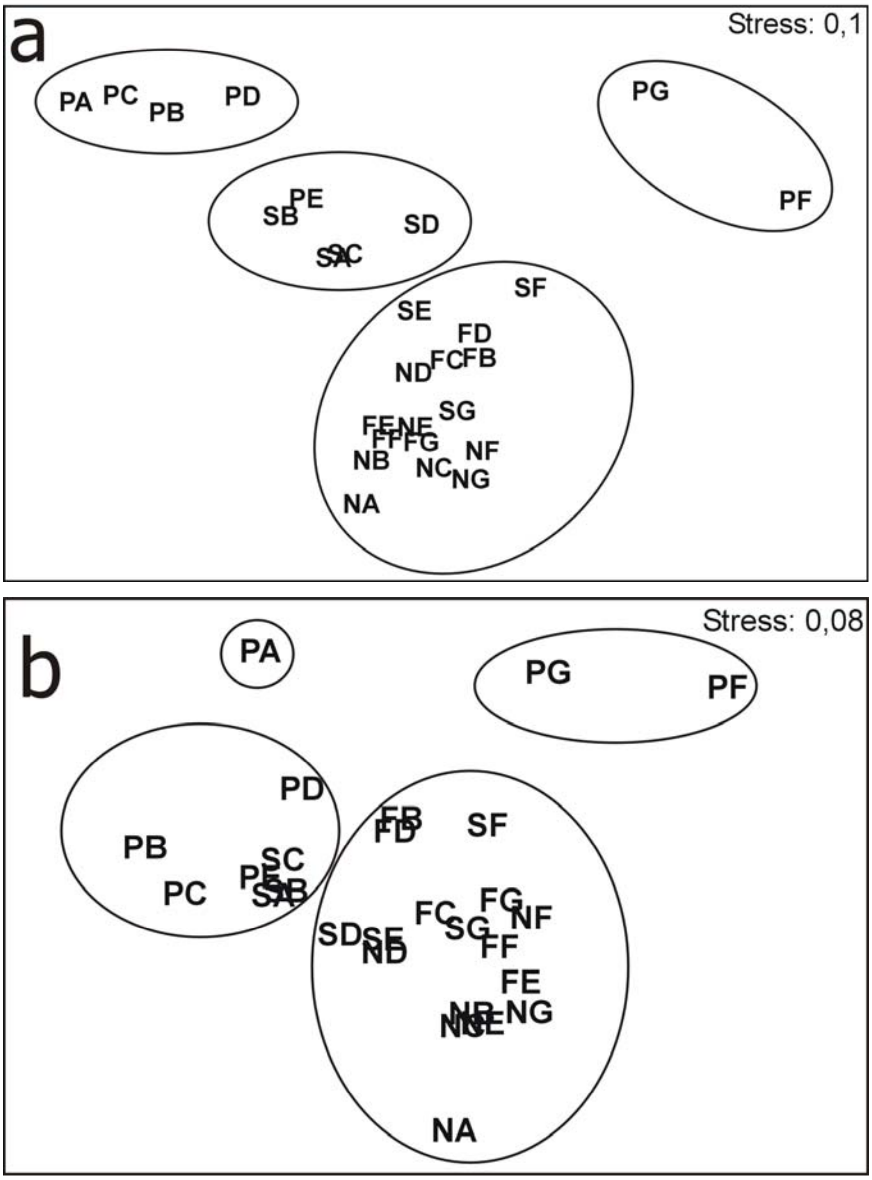
According to BioEnv, the variable that best explained the community pattern was the AVS with a correlation of 0.169, followed by ammonia with a correlation of 0.163 and C/N with a correlation of 0.111. The highest correlation was 0.303 and was obtained using these three environmental variables together.

Figure 7. Time variation of feeding guilds abundance for each station (mean, n=4).



SIMPER was applied using the individual abundance matrix, comparing every station and the different surveyed times. The analysis showed that polychaete families, Capitellidae, Spionidae and Nereidae (in this order), were the most representative taxa of the impacted stations when fish-farming was in operation. The taxa which best represented the reference station and impacted stations after abatement were the order Gammaridea, *Apseudes latreillei* (Tanaidacea) and the polychaete family Onuphidae. The polychaete family Sabellidae also seemed to be useful as an indicator of non-polluted areas, although to a lesser extent.

Figure 8. a) MDS based on species at the different stations in different surveys. The MDS results have been grouped according to the cluster results, each assemblage showing approximately 60% or greater similarity. b) MDS based on feeding guilds of the different stations in different surveys. The MDS results have been grouped according to the cluster results, each assemblage showing approximately 75 % or greater similarity. The first letter of each point corresponds to the station (F, N, S and P) and the second one corresponds to the time. A: October 2001, B: January 2002, C: May 2002, D: October 2002, E: April 2003, F: July 2003, G: November 2003



DISCUSSION

This work is the first study in the Western Mediterranean related with benthic recovery after open sea fish-farm abatement. Its relevance is based on the comparison of species abundance, trophic groups and a benthic index (AMBI) to assess benthic recovery.

As regards the physico-chemical parameters, AVS was the most suitable chemical parameter for assessing and monitoring eutrophication in fish farming, as Pawar et al. (2002) also found. Redox potential was the second best variable, although in the BioEnv analysis, ammonia and C/N seemed to be more robust variables. This is because this type of analysis does not permit evaluation of the whole redox profile. Even though, the redox potential can on some occasions lead to misleading conclusions. The first abandoned station (N) did not seem to have recovered because the redox potential on the surface was still strongly negative, perhaps due to the existence of dead mats of the seagrass *Posidonia oceanica*, which would favour the accretion of limes and clays. However, dead mats may also act as a substrate and encourage benthic faunal establishment. BioEnv as well as ANOVA showed that the measured environmental parameters differed significantly in time and space, and presented low correlations with the macrofauna. Clearly, environmental parameters on their own could not explain the succession and recovery trends, although some parameters did help to throw light on the status of the benthic ecosystem.

The benthic index (AMBI) has been shown to be a useful tool for assessing organic enrichment, since it provides a quite accurate picture of the real situation.

The potential recovery of an area after organic enrichment depends on the abiotic and biotic factors that determine the benthic community and on the hypoxia and anoxia tolerance of the species involved (Diaz and Rosenberg, 1995). Such recovery seems to follow defined patterns (Pearson and Rosenberg, 1978; Diaz-Castañeda et al., 1993). Going against the classical paradigm of succession as a continuum, Karakassis et al. (1999) introduced a different perspective for the recovery process. These authors considered that recovery has the same ‘‘final point’’ as Pearson and Rosenberg (1978) proposed, but with a highly fluctuating rate that produces forward and backward movements, due to stochastic secondary perturbances in the ecosystem.

In our experiment, succession did not follow a predetermined temporal pattern according to trophic structure and MDS analysis. There was a considerable spatial variation, although each station seemed to tend to the same successional end. This observation coincides with that of Rosenberg et al. (2002) who found that the pioneering and mature benthic successional stages were predictable but not the intermediate stages.

Less stable habitats usually recover more quickly than stable habitats (Dernie et al., 2003), and so this kind of environment studied was expected to make a rapid recovery. Since the impact of open sea fish farms is restricted to a relatively small perimeter around the cages, benthic recovery is made possible by the recolonization of nearby non-affected areas.

The recovery rate and the way in which it takes place can only be understood by taking into consideration the great physico-chemical variability in environmental parameters (Dernie et al., 2003). Furthermore, basic characteristics of the water area such as topography, hydrodynamic conditions, water turbidity presence or absence of a sharp temperature stratification and water exchange patterns should also be considered (Kraufvelin et al., 2001). These parameters give to each location an almost unique configuration that makes it difficult to compare with others. However, we propose that not only environmental variability influences the time of recovery, but that trophic structure (feeding guilds) may also play a major role in recovery processes.

MDS analysis and the Shannon–Wiener index pointed to a clear pattern of recovery in the impacted stations. Both analyses suggested that station N and S had fully recovered by the last survey, while station P had not. Even so, no indicators of heavy organic pollution, such as Capitellidae, Spionidae or Nereidae families, were found in the sampled community during the last survey. This fact leads us think that station P followed a way of succession that differed considerably from that followed by the other impacted stations.

Our results suggest that AMBI is appropriate for assessing organic pollution levels but not for evaluating community structure status.

MDS analyses based on abundance and on feeding guilds were performed because species diversity does not always necessarily reflect functional diversity. Ecosystem processes are affected by the functional characteristics of organisms involved, rather than by taxonomic identity (Loreau et al., 2002). The number of functionally different roles represented in an ecosystem may be a stronger determinant of ecosystem processes than the total number of species, per se (Tilman et al., 1997; Hector et al., 1999). The fact that both MDS-A and MDS-F showed a similar ordination of successional changes indicated that there were differences in species composition and abundance but also in trophic structure (feeding guilds). Functional groups provide a perspective of the ecological processes and the status of the ecosystem. MDS analysis based on functional groups, such as feeding guilds, could be used as a complementary tool for assessing organic enrichment impact.

The analysis based on feeding guilds through time provided useful information concerning organic matter perturbation. Fish farm impacted stations showed an unbalanced feeding guild ratio, where the surface deposit feeders were the prevailing group. However, the reference station remained with a relatively constant feeding guild ratio throughout the experiment. Feeding guild ratio stability seems to be a logical expectation for non-disturbed ecosystems with considerable diversity and species redundancy. This fact coincides with the view of Andrew and Hughes (2005), who found that feeding guild ratios between insects living in the same tree species was constant in pristine environments, even at different latitudes.

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