UNIVERSITAT DE BARCELONA

FACULTAD DE BIOLOGÍA
Departamento de Ecología

PROGRAMA DE DOCTORADO EN CIENCIAS DEL MAR
Bienio 2003-2005

TESIS DOCTORAL

Estudio y desarrollo de indicadores biológicos para evaluar el alcance espacial de vertidos procedentes de granjas marinas

Presentada por

María García Sanz

Para optar al título de Doctor en Biología

DIRECTORES:

Marta Pérez Vallmitjana

Juan Manuel Ruiz Fernández

Barcelona, diciembre de 2009

A mis padres,
A Nando

Agradecimientos

Esta es la parte de la tesis que más me apetecía redactar, tanto por poder expresarme a mi aire, cosa que se agradece después de lo encorsetado de la tesis, como por tener un recuerdo escrito de lo que me ha venido ocurriendo en estos años y sobre todo de las personas que han estado acompañándome. Esto es especialmente importante si se tiene memoria de pez, como la aquí presente, y se necesita algo de ayuda para echar marcha atrás en el tiempo. Aviso de antemano de que a quien no le guste lo de los sentimentalismos, que pegue un bote a esta sección, porque no pienso evitarlos!! Así que sin más rollos comienzo con mis agradecimientos. Nunca pensé que llegaría este momento….!!!!

Mi primer gracias va a mis directores de tesis, Marta y Juanma por su ayuda, apoyo, motivación y ganas de hacer las cosas bien. He aprendido a trabajar con vosotros. Me habéis enseñado las tripas de la ciencia y me habéis dado herramientas para poder enfrentarme a todos los retos que se me han puesto por delante. Gracias a vosotros he ganado confianza en mi misma, he sabido enfrentarme a mis miedos y he escrito una tesis, que no es poco!! Gracias por guiarme tan bien por este camino y gracias por intentar trasmitirme vuestra pasión por lo que hacéis. También quería agradecer a Javier, mi "casi-director", lo que he aprendido en su grupo todo este tiempo. Vine a Barcelona guiada por su nombre, y he comprobado que no andaba mal encaminada. A parte de que me voy de Barcelona admirando aun más su trabajo, le tengo que agradecer mi introducción en el mundo de la esgrima, aunque el resultado dejase mucho que desear.

Gracias también a mi súper grupo de trabajo. Gracias Oriol, Izaskun, Neus, Silvia y a "mi Jordi". He visto como crecía este grupillo y la verdad es que sois unos craks. Me habéis ayudado un montón con esta tesis. Me habéis resuelto mil y una dudas y me habéis hecho compañía en un cuartucho del que no se si podré olvidarme algún día!! No podría haber tirado muertos al agua sin ti, Oriol. Decirlo aquí queda un poco feo, pero es la realidad. Menos mal que podemos demostrar que no es delito..!! Ni habría cogido alguitas tan entretenida sin ti,

Izaskun. Nos hemos perdido por el delta, por Menorca, hemos hecho el hippy en “fragoneta”, hemos dado lecciones de conducir, con trompos incluidos, hemos pescado, buceado y hemos convivido en sitios de los más dispares; desde chalets “casa pepe” a mini bungalows o micro habitaciones de hotel… Menos mal que siempre caía alguna fideua para alegrar el cuerpo después de muestrear en fango apestoso, con plaga de anémonas y con 0 ºC. Madre mía, parece que no, pero hemos vivido un montón de cosas juntos. Sois mis compis preferidos!!!!

Volviendo a lo del cuartucho, creo que tampoco podrán olvidarse de él un montón de compañeros como Carles, Gemma, Iraima, Mary, Dani, Biel, Blanca, Raúl, Cesc, Eusebi, Julio, Marta, Lidia, y otros cuantos que me faltan por nombrar. Gracias por las charlas entre pasillos, por el interés, por los momentos que hemos compartido y por la ayuda que me habeis prestado cuando la necesitaba y cuando no. Mil gracias. También me gustaría agradecer la ayuda que me han ofrecido Pilar Fernández, Belén, Ana, Pilar Teixidor, Pili, Santi Matas, Paco y Jaume de diferentes serveis de la universidad. Me habéis ayudado en disoluciones, cálculos de amonios, concentraciones, ppm, moles, tantos por mil de isótopos, me habéis contado truquillos y me habéis escuchado en cada ocasión que os he ido a pedir algo.

Me gustaría recordar aquí las campañas en las que he participado y que me han permitido conocer un poquito de mundo. He descubierto la costa catalana de la que me he quedado enganchada. He conocido Murcia, Tarragona y Canarias con las campañas de la Jacu. Fueron mis primeras campañas donde conocí a Juanma, Rocío, Lolo y Salva entre otros. En ellas aprendí que no se puede dormir con la ventana abierta en Canarias y que hay que llevar remos en la barca por si acaso!! También conocí al personal de la granja de L´Ametlla de Mar Xavier Sabater, Pepito, Yuval y cia… Fue toda una experiencia para los que estuvimos allí. Yo por lo menos nunca podré olvidar la cabina de ese barco…Gracias por todo. Después de estas campañas he estado en Córcega con Mian y Begoña en furgo y con huelga incluida. Y por último este trabajo me ha permitido conocer sitios como Malta, Hamburgo, Bremen, Lecce y Niza gacias al AquAgris. Allí he conocido un montón de gente de reunión en reunión con la que he aprendido un montón y que me ha echado una mano a lo largo de la tesis. Gracias.

Y ahora viene una retaíla de nombres sin los cuales no podría haber hecho nada de lo hay aquí escrito, no tanto por el apoyo directo (que también) sino por lo importante que resultan en mi vida.

Laurita, te quiero!!! Eres mi compañera del alma, mi súper amiga y nada de esto hubiese sido lo mismo sin ti. Comenzamos juntas y acabamos juntas. Somos las mejores!! Ya te dije en el dea, que era un placer compartir cualquier cosa contigo y en esta tesis hemos compartido millones de cosas, de curro y de no curro, buenas y malas, divertidas y aburridas. En fin que ha habido de todo y espero que siga habiéndolo allá por donde estemos…

Gracias Samar, por un montón de cosas. Hemos aprendido inglés chapurrero juntas. Menudas sesiones de traducción de artículos que nos hemos dado! Eso si, siempre rodeadas de mosquito tigre y con vistas a Collserola. Un lujo! También te tengo que agradecer tu ayuda en las campañas de Cataluña y Murcia. Nos lo pasamos muy bien en las dos, a pesar de que yo estaba hecha un manojo de nervios. Pero, bueno, para tranquilizarnos siempre podíamos perdernos durante tres horas por lo pagan buscando un portal fantasma… Gracias por tu compañía en nuestro viajecillo a Madrid donde descubrimos lo que es un isótopo y para todo lo que sirven… hasta para saber si un jamón es de bellota o no! Que cosas…

Massimo, que fuerte, que fuerteeee!!! Que esto se acaba!!! Y parecía que no tenía fin… Muchas gracias por estra siempre ahí. Para charlar, comer, salir, rescatarme, escucharme, bailar, acompañarme de noche a cuidar alguitas… y un sin fin de cosas más. Animo en el final Massi, que tu puedes!!

Beatrichu, mi oso panda emigrado a china… Me has echado una mano en lo que te he pedido y siempre me has dado más de lo esperado. Me sigues sorprendiendo como persona, y eso que para mí ya eres la mejor. Gracias por todo.

Paula, Patri, Mariajo, Cris, Champi, Laura, mis amigas desde siempre. Somos una piña piñonera, y aun siendo tan distintas entre nosotras, siempre me habéis comprendido y apoyado en lo que hago. Me habéis escuchado quejarme y alegrarme y os he metido unos rollos tremendos con mis atascos y baches. Os quiero un montón y me hacéis la vida más bonita. Gracia por estar ahí, siempre.

Gracias a mis dos familias de Barcelona. La primera, Xavi, Cris y Laura y a la segunda, Sergi Tito e Izaskun. La convivencia con vosotros ha sido una super experiencia en todos los sentidos. Imposible repetir, imposible mejorar…

Anna, Nacho y Carlos, me encanta como ha quedado la tesis. Viva el diseño!! Gracias por ayudarme a darle forma a todo este montón de letras y hacerlo agradable a la vista. Me encanta!!

Gracia a mi Tizón porque se ha pasado media tesis conmigo. Es mi más fiel admirador y no se me ha separado ni un minuto en todo el tiempo que me he pasado redactando en casa… Guaauuuuuuu!

Gracias a mis padres por ciento de miles de cosas… por apoyarme, quererme, cuidarme, aguantarme y sobre todo por ser como sois. Os quiero muchísimo.

Gracias a mi familia porque aunque siempre han estado lejos hacen que les sienta cerquita.

A Leito y Miguel por enseñarme el mar por fuera.

A Paula y Mariano por enseñarme el mar por dentro.

Y por último mil gracias Nando. Por una sinfín de cosas buenas que has traído a mi vida. Por fin vamos a saber lo que es vivir sin una tesis rondando por nuestras cabezas. Es una suerte poder cerrar este capítulo de mi vida y poder descubrir lo que vendrá al lado tuyo. Te quiero.

Y ya solo me queda escribir una última cosa, la que llevo deseando escribir desde hace ya un buen tiempo y por la que todo el mundo me pregunta, asi que ahí va….

FIN!!!!

ÍNDICE / Contents

INTRODUCCIÓN GENERAL

Introducción general

La acuicultura marina basada en la cría o engorde de organismos acuáticos, representa una alternativa para abastecer de pescado a la población mundial, debido a la sobreexplotación y el agotamiento de los caladeros de las principales especies comerciales que han conducido a la crisis actual que sufren las pesquerías en todo el mundo (Pauly y Christensen, 1995). La población mundial se duplicó entre 1960 y 2000 y se estima que llegue a los 7 billones de habitantes en el 2012 de acuerdo con la Base de Datos de la Oficina Internacional de Censos (IDB). La producción de la pesca de captura cesó de crecer a mediados de la década de 1980, fluctuando desde entonces en torno al mismo valor (90-95 millones de toneladas). En cambio, el sector acuícola ha incrementado su producción continuamente en los últimos 50 años, con una tasa de crecimiento anual de casi el 7 %, compensado los efectos del estancamiento de la producción de la pesca de captura y el aumento de la población mundial (Figura 1).

Dentro del sector acuícola, el engorde y producción de organismos marinos cultivados en jaulas flotantes es uno de los segmentos que presenta una mayor tasa de crecimiento a nivel mundial, a pesar de ser una práctica relativamente nueva (Tacon y Halwart, 2007). Esta información se extrae de estudios realizados a nivel regional ya que no existe información estadística oficial sobre el total de la producción mundial o sobre el crecimiento de la acuicultura en jaulas en conjunto (Halwart et al., 2007).

Este tipo de acuicultura intensiva se inició en Noruega en la década de 1970 con el surgimiento y desarrollo de la cría de salmón (Beveridge, 2004) expandiéndose rápidamente a mediados de la década de 1980 a los países del sur de Europa, especialmente a España y Grecia. En el Mediterráneo la producción de la acuicultura en jaulas se ha incrementado progresivamente en los últimos 10 años, desde 34.700 toneladas en 1995 hasta 137.000 toneladas en 2004, con un crecimiento anual promedio del 17 por ciento (FAO/FIDI, 2006). Las

principales especies que se cultivan en el Mediterráneo son la lubina (*Dicentrarchus labrax*) y la dorada (*Sparus aurata*) y según datos de 2004 representan alrededor del 85 % de la producción total en jaulas (Cardia y Lovatelli, 2007). El engorde del atún rojo del Atlántico (*Thunnus thynnus thynnus*) en grandes jaulas flotantes es una modalidad reciente dentro de este subsector de la industria acuícola. Esta actividad comenzó a desarrollarse a mediados de la década de 1980 (España), pero la expansión más rápida de este tipo de cultivo se produjo a mediados de los años 90. La producción total registrada oficialmente en el Mediterráneo en 2003 fue de aproximadamente 19.000 toneladas (FAO/GFCM/ICCAT, 2005).

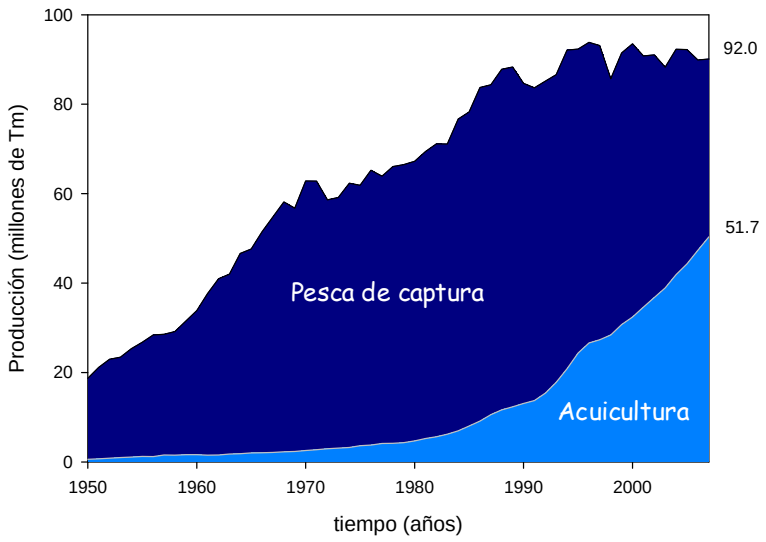


Figura 1. Evolución de la producción (pesca y acuicultura) en el mundo en el periodo 1950-2006. Plantas marinas no incluidas (FAO, 2008)

El cultivo intensivo en jaulas se realiza actualmente en estructuras de polietileno de alta densidad (PEAD) (Foto 1) con una malla de luz de entre 15 y 20 mm para el cultivo de lubina y dorada y de alrededor de 100 mm para el cultivo de atunes (Foto 2), lo que permite un flujo

continuo de agua a través de la instalación y una descarga continua de nutrientes y materia orgánica originados por la actividad del cultivo.

Foto 1. Jaulas marinas de polietileno de alta densidad (PEAD) destinadas al cultivo de pescado.

Este tipo de aportes representa una importante fuente de contaminación susceptible de causar impactos tanto en la columna de agua como en el bentos, especialmente si el flujo de estos compuestos hacia el ambiente supera la capacidad de asimilación de los ecosistemas marinos. Por lo tanto, el control de la dispersión de los vertidos acuícolas en el medio marino y la evaluación del impacto ambiental de los aportes orgánicos e inorgánicos procedentes de dicha actividad representan actualmente uno de los principales retos para el desarrollo de una gestión sostenible de la acuicultura, compatible con la conservación de los ecosistemas marinos costeros y su biodiversidad (Wu, 1995; GESAMP, 1996; Karakassis, 1998). No obstante, la cuantificación de los efectos de la acuicultura en los ecosistemas marinos costeros, y el desarrollo de las herramientas adecuadas para su evaluación en el espacio y en el tiempo, son problemas que no han sido todavía resueltos de forma eficaz y práctica.

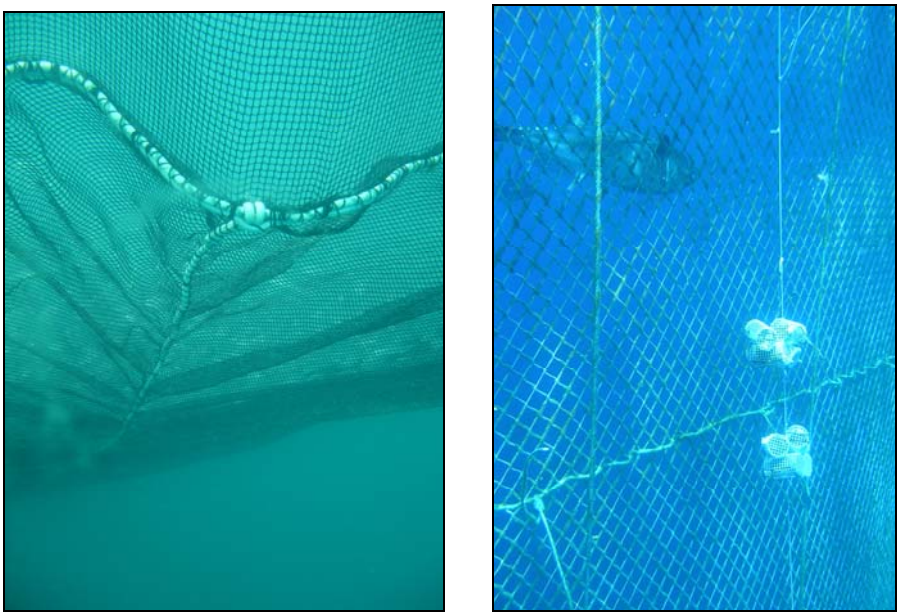


Foto 2. A la izquierda, detalle de red de una jaula de cultivo de dorada. A la derecha, detalle de red de una jaula de engorde de atún.

Vertidos derivados de la acuicultura en jaulas

La principal entrada de nutrientes y materia orgánica en el medio se produce durante de el proceso de alimentación y a través de los desechos generados por el metabolismo de los peces cultivados (Figura 2). Los nutrientes y la materia orgánica procedentes de estos procesos pueden liberarse al medio tanto en forma disuelta como particulada siendo, como norma general, el sedimento el recipiente principal del desecho particulado y la columna de agua el recipiente para el desecho soluble y material particulado más fino, aunque existe intercambio de materia entre el sedimento y la columna de agua mediante procesos de remineralización y resuspensión.

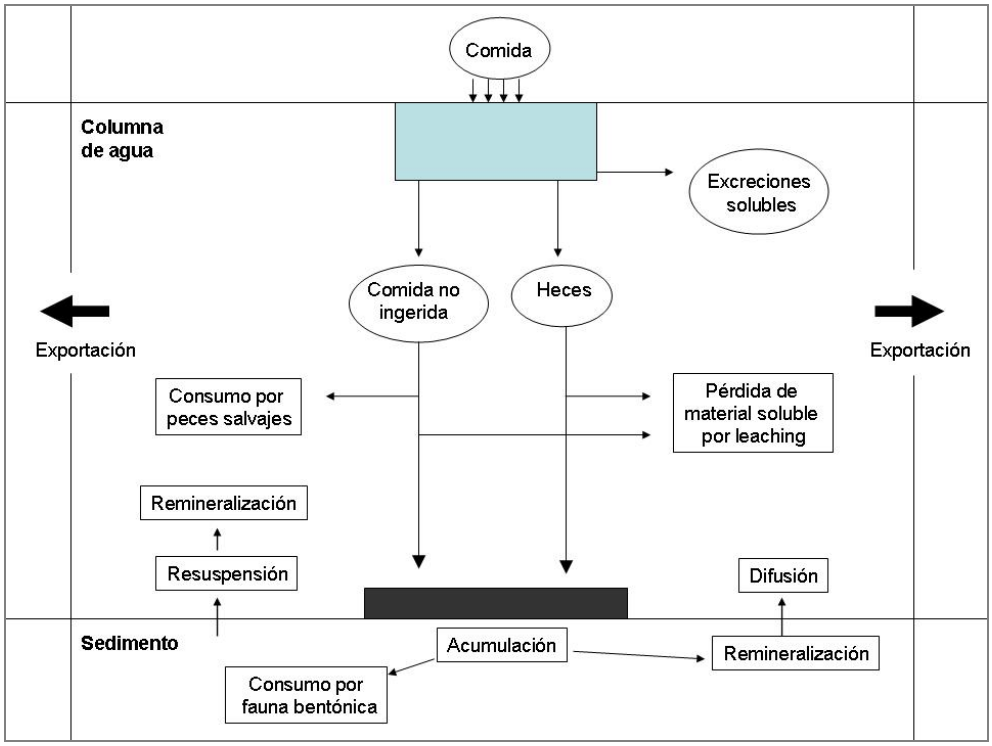


Figura 2. Destino de los vertidos procedentes de la acuicultura en las inmediaciones de una granja marina

La fracción disuelta en forma de compuestos nitrogenados representa la parte más importante del vertido derivado del metabolismo de los peces cultivados (Tabla 1) (Islam, 2005; Roque d´Orbcastel et al., 2008; Merino et al., 2007). En el caso del cultivo de túnidos esta fracción disuelta es especialmente importante debido a que los atunes son engordados a base de pescado fresco o descongelado y a que presentan una tasa de conversión de alimento (FCR)[1] elevada (FCR: 7.68; Aguado et al., 2006), en comparación con la de dorada o lubina (FRC: 1.5-

[1] El *factor de conversión de alimento* (FCR) representa la eficiencia de utilización del alimento y queda definido como la razón entre la cantidad de alimento suministrado y el crecimiento en peso del individuo.

2.5; Lupatsch y Kissil, 1998). La particular fisiología de esta especie (endotérmica y nadadora continua) y su comportamiento tanto alimentario como reproductor (largas migraciones) hace que tenga una gran demanda energética (suministrada principalmente por el metabolismo de proteínas) y una baja eficiencia de conversión de alimento ya que la mayor parte de la energía es usada para mantenimiento y solo una parte pequeña se invierte en crecimiento (Graham y Dickson, 2001; Korsmeyer y Dewar, 2001).

Tabla 1. Comparación de los vertidos diarios de N total y P total (mg nutrientes kg pescado^{-1} día^{-1}) derivados del metabolismo de dorada (*Sparus aurata*), lubina (*Dicentrarchus labrax*) y atún (*Tunnus thynnus thynnus*).

	N particulado	N disuelto	P particulado	P disuelto	Referencias
Sparus aurata	51.6	182.4	31.3	11.3	Lupatsch y Kissil (1998)
Dicentrarchus labrax	62.3	382.9	21.6	14.4	Lemarié et al., (1998)
Thunnus thynnus thynnus	49.4	694.2	70.0	51.7	Aguado et al., (2006)

La fracción de mayor tamaño del material particulado de los efluentes acuícolas sedimenta y se acumula en el sedimento dentro de un área prácticamente circunscrita a la superficie ocupada por las jaulas de cultivo o, como mucho, hasta pocos cientos de metros desde el perímetro de las mismas (e.g. Cromey et al., 2002). Las poblaciones de peces salvajes concentradas fuera de las jaulas, junto con los procesos de resuspensión del sedimento, pueden contribuir a dispersar el material particulado a distancias mayores que las predichas por modelos numéricos basados en el tamaño de las partículas y su densidad (Sarà et al., 2004; Fernández-Jover et al., 2007). La

fracción disuelta y el material particulado más fino suspendido en la columna de agua es transportado hasta distancias mucho mayores, del orden de kilómetros (Tsapakis et al., 2006; Dolenec et al., 2007; Sarà, 2007). La extensión de la dispersión de las diferentes fracciones del vertido dependerá de las características del cultivo (producción, especies, alimentación, etc.), del efluente resultante y del régimen local de corrientes, así como de la época del año, liberándose cantidades mayores en los meses de verano debido a que las tasas de alimentación son más altas en esta época (Sarà et al., 2006; Roque d´Orbcastel y Blancheton, 2006; Aquatreat, 2007).

Impactos de los vertidos de la acuicultura en jaulas

Entre los impactos más destacados asociados con los vertidos procedentes de la acuicultura cabe mencionar la eutrofización, la anoxificación de los sedimentos y la alteración de la estructura y funcionamiento de las comunidades biológicas (Chua, 1992; Wu et al., 1995; Samocha y Lawrence, 1997; Karakassis, 2000; Holmer et al., 2008). Los ambientes marinos costeros del Mediterráneo se caracterizan por el marcado carácter oligotrófico, e incluso ultraoligotrófico, de sus aguas (Margalef, 1989). Es previsible, por tanto, que las comunidades biológicas estrechamente adaptadas a tales condiciones sean especialmente vulnerables a los cambios ambientales causados por el incremento de las concentraciones de nutrientes y de materia orgánica en el medio. Dichos cambios ocurren de forma especialmente intensa en los sedimentos bajo las jaulas e inmediaciones de las mismas causando alteraciones significativas en las comunidades microbianas (Vezzulli et al., 2002), en la infauna (Karakassis et al., 2000; Mirto et al., 2002) y en las angiospermas marinas (Delgado et al., 1997; Ruiz et al., 2001; Cancemi et al., 2003; Apostolaki et al., 2009) asociadas a ambientes marinos costeros del Mediterráneo.

Las praderas de fanerógamas marinas, y en particular las de *Posidonia oceanica* en el Mediterráneo, son un claro ejemplo de ecosistemas sensibles al impacto de los vertidos de granjas marinas en el medio. De hecho, numerosos trabajos han documentado la alteración y regresión de praderas de *P. oceanica* bajo la influencia de los vertidos orgánicos de granjas marinas (Delgado et al., 1999; Pergent et al., 1999; Ruiz et al., 2001; Invers et al., 2003, Cancemi et al., 2003, Homer et al., 2008; Apostolaki et al., 2009) (Foto 3).

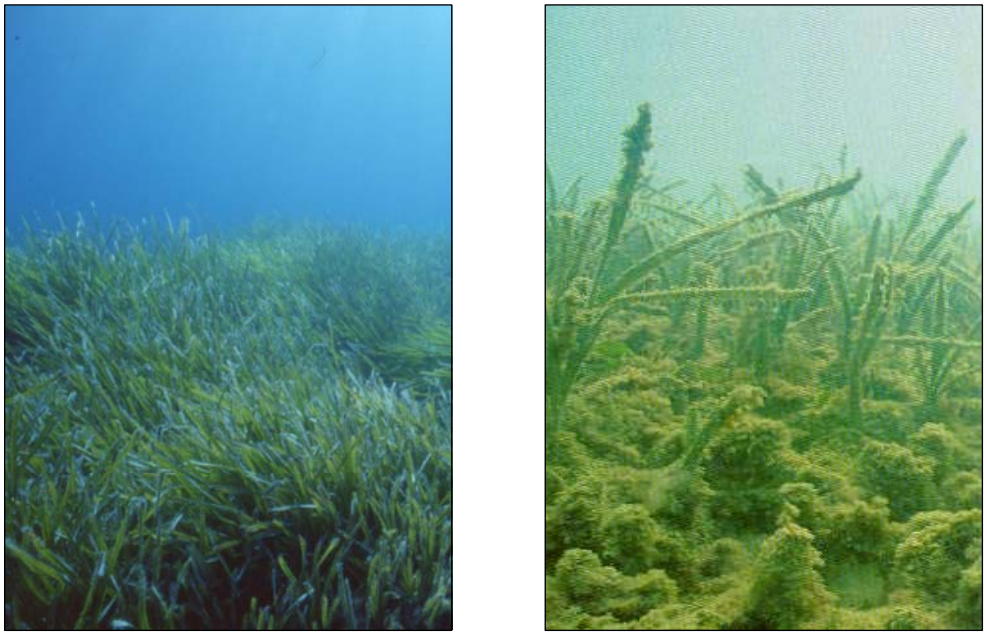


Foto 3. Pradera de *Posidonia oceanica* en buenas condiciones ambientales vs pradera expuesta a vertidos de instalaciones de acuicultura. Foto: Enrique Ballesteros.

Los vertidos de las granjas marinas modifican las características de la columna de agua y del sedimento (e.g. Holmer et al., 2007), lo que puede alterar la estructura de las praderas y comprometer la supervivencia de las mismas a través del incremento de la carga de epífitos y de la presión de herbívoros (Delgado et al., 1997; Ruiz et al., 2001, Leoni et al., 2006) o de la alteración del metabolismo de la planta (Pérez et al., 2007; Frederiksen et al., 2008; Pérez et al., 2008).

La indiscutible importancia ecológica y económica de ésta y otras comunidades de elevada sensibilidad ecológica, ha motivado que el control de la dispersión de los aportes orgánicos de las granjas marinas en el ecosistema marino costero sea actualmente una de las principales preocupaciones por parte de técnicos y gestores responsables de la ordenación de esta actividad en el litoral (SGPM 2001). La mayoría de los impactos documentados en praderas de *Posidonia oceanica* se han producido en ambientes someros con cierto grado de protección, donde la dispersión de los vertidos es limitada y la sedimentación del material particulado es especialmente intensa. Por esta razón, durante la década de los 1990 la mayor parte de las instalaciones acuícolas han sido progresivamente trasladadas a ambientes más profundos y alejados de la costa (acuicultura *off-shore*) donde las condiciones hidrodinámicas facilitan una mayor dispersión del vertido y, supuestamente un menor impacto sobre las comunidades bentónicas (Basurco y Larrazabal, 2000; Maldonado et al., 2005). No obstante, la capacidad para caracterizar y cuantificar la dispersión de los vertidos acuícolas en estos ambientes más dispersivos es más limitada debido a la elevada variabilidad espacio-temporal del comportamiento de los nutrientes en la columna del agua, así como a la menor accesibilidad y disponibilidad de bioindicadores en estas zonas más profundas. De hecho, prácticamente todos los estudios realizados en la columna de agua en sitios con suficiente circulación de agua, no detectan cambios en la concentración de nutrientes o detectan un aumento localizado de nutrientes o materia orgánica particulada en la columna de agua (Nordvarg y Johansson, 2002; Soto y Norambuena, 2004). Solo en algunas piscifactorías localizadas en bahías cerradas o sitios muy someros, varios autores encontraron aumentos en la turbidez alrededor de las jaulas, entre

otros efectos, debido a una alta densidad de fitoplancton y el desarrollo bacteriano (Pitta et al., 1999; Karakassis et al., 2001; Sakami et al., 2003). La aparente ausencia de nutrientes en la columna de agua alrededor de las piscifactorías ha sido atribuida a una rápida dilución o dispersión de los nutrientes (Pitta et al., 2006) y a una rápida transición hacia niveles más altos de la cadena trófica, llevando a un aumento tanto en la abundancia de peces salvajes alrededor de piscifactorías (Machias et al., 2004, 2005) como en la producción general de las pesquerías en el área (Machias et al., 2006). Sin embargo, estos resultados pueden estar más relacionados con la capacidad limitada de los métodos e indicadores empleados tradicionalmente en la evaluación de la dispersión de los vertidos acuícolas, más que con la ausencia de nutrientes procedentes de dichos vertidos en el medio tal y como indican un número cada vez mayor de estudios basados en el empleo de diferentes tipos de bioindicadores (e.g. Dalsgaard y Krause-Jensen, 2006; Lin y Fong et al., 2008).

Uso de bioindicadores

Como se ha comentado anteriormente, las técnicas analíticas estándar que usan variables físico-químicas para el seguimiento de la calidad de las aguas alrededor de cultivos marinos en jaulas proporcionan generalmente muy poca información sobre la calidad ambiental de las aguas a causa de la variabilidad espacial y temporal asociada con estas medidas (Lyngby, 1990; Wolanski et al., 2000; Karakassis et al., 2001). Por este motivo, en los últimos años se ha fomentado el uso de bioindicadores, es decir, especies o comunidades de organismos cuya presencia, comportamiento o estado fisiológico presenta una estrecha correlación con determinadas circunstancias del entorno, con el fin de evaluar la calidad de las aguas alrededor de cultivos marinos en jaulas.

El empleo de organismos bentónicos como indicadores de la influencia de la actividad humana sobre el medio representa una alternativa adecuada y eficaz al uso de métodos más tradicionales. Cambios en la abundancia de determinados taxones y en especies de fauna se miden rutinariamente para ser expresados en forma de índices biológicos, los cuales se aplican extensivamente para evaluar la respuesta de las comunidades bentónicas a los impactos de los vertidos orgánicos de la acuicultura (e.g. Borja et al., 2000; Simboura y Zenetos, 2002; Aguado et al., 2007). Sin embargo, su aplicación es costosa en tiempo y dinero debido al difícil acceso al sitio de muestreo (requiere empleo de dragas) y a la separación y clasificación taxonómica de las muestras. Además, la información aportada se limita a un estado de alteración tardío y no es del todo coherente entre los diferentes índices biológicos empleados. Por otra parte, el uso de macrófitos bentónicos (algas y angiospermas marinas) como indicadores de la influencia de vertidos derivados de actividades humanas no se lleva actualmente a cabo de forma rutinaria, aunque se están empezando a utilizar para la evaluación del estado de las aguas costeras en cumplimiento de la Directiva Marco del Agua (DMA 2000/60/EC) (Panayotidis et al., 2004; Ballesteros et al., 2007; Romero et al., 2007). Los macrófitos bentónicos son considerados excelentes indicadores debido a su capacidad de obtener y asimilar nutrientes, a su larga vida (en contraste con el fitoplancton, por ejemplo, así la evidencia permanece más tiempo en el sistema), así como por el amplio conocimiento documentado sobre los efectos de estos nutrientes en su distribución, estructura y fisiología (Lyngby, 1990; Alamoudi, 1994; Horrocks et al., 1995; Udy y Dennison, 1997; Jones et al., 2001).

Sin embargo, la distribución limitada y heterogénea de las poblaciones de macrófitos bentónicos impide su empleo como bioindicadores en zonas profundas y sobre amplias escalas espaciales. Esta limitación ha sido superada mediante el empleo de los macrófitos a modo de bioensayos activos (Costanzo et al., 2001; Dalsgaard y Krause-Jensen 2006), en los que se ha demostrado que el análisis de tejidos de macrófitos incubados durante cortos periodos de tiempo (días) es capaz de aportar información fiable sobre la forma y extensión de los gradientes de nutrientes originados por fuentes antropogénicas. Sin embargo, en determinadas circunstancias

la relación entre los 'pools' internos de la planta y el régimen externo de nutrientes no es directa, bien debido a un efecto de dilución por el crecimiento del tejido vegetal o bien por la existencia de varias fuentes diferentes de aportes de nutrientes al medio, puntuales o difusas. Esta dificultad se ha visto solventada, para el caso del nitrógeno, por el empleo de isótopos estables del N, que permiten identificar en los macrófitos la influencia de N procedente de fuentes específicas.

El uso de la señal $\delta^{15}N$ medida en tejidos de macrófitos para identificar fuentes de nitrógeno está basado en el hecho de que diferentes fuentes de nitrógeno tienen distinta composición isotópica (Tabla 2) (Heaton, 1986), y en la habilidad conocida de los macrófitos de tomar, asimilar y almacenar los nutrientes en exceso del medio (Lyngby et al., 1990). En condiciones naturales, la señal isotópica de los tejidos de algas y angiospermas marinas se aproxima a cero ya que el N disponible es de origen atmosférico. Los aportes de fertilizantes químicos (i.e. utilizados en agricultura) al medio marino costero no causan una desviación de estos valores pues también son de origen atmosférico. Por el contrario, los aportes orgánicos de origen animal (vertidos urbanos y acuícolas) se reflejan en un aumento de la señal isotópica de los tejidos de macroalgas, fanerógamas y manglares (Wada et al., 1987; Grice et al., 1996; Udy and Dennison, 1997). Estos elevados valores no son tanto debidos a la señal isotópica de las fuentes (excrementos animales) sino más bien a los procesos de fraccionamiento isotópico que experimenta el N de dichas fuentes en el medio (Fry, 2006). Efectivamente, la elevada señal isotópica del pool de N de vertidos urbanos se debe a la pérdida del isótopo ligero del sistema en los procesos de volatilización del amonio y subsiguiente nitrificación (Lahjta y Michener 1994; Cole et al. 2005). Estos procesos de fraccionamiento isotópico también explican el incremento de $\delta^{15}N$ entre 3 y 4 ‰ entre niveles tróficos (Hobson et al., 1996), circunstancia que se ha aprovechado para determinar la posición trófica de los organismos que forman parte de una comunidad determinada.

Así pues, la combinación de macrófitos, bioensayos y análisis de los isótopos estables

del N ha demostrado ser una herramienta poderosa y eficaz para caracterizar gradientes espaciales de nutrientes asociados a vertidos de aguas residuales urbanas en ambientes marinos costeros (Costanzo et al., 2001; Deutsch y Voss, 2006). Sin embargo, a pesar del enorme potencial de este tipo de aproximaciones metodológicas, su desarrollo y aplicación para determinar el alcance espacial de los vertidos de la acuicultura ha recibido escasa atención hasta el momento (Lin y Fong, 2008).

Tabla 2. Rango de valores de $\delta^{15}N$ para las fuentes más comunes de compuestos nitrogenados en el medio hídrico.

$\delta^{15}N$ (‰)	Origen de los compuestos nitrogenados
0	N_2 atmosférico
1-2.5	Fertilizantes químicos
9-14	Aguas Residuales
9-11	Residuos acuícolas
10-30	Residuos Ganaderos

Objetivos y estructura de la tesis

El objetivo principal de esta tesis ha sido el desarrollo de herramientas adecuadas que permitan estimar el alcance espacial de los vertidos procedentes de las granjas marinas flotantes dedicadas al cultivo de peces, basadas en la utilización de los macrófitos bentónicos (macroalgas y fanerógamas) como indicadores del régimen de nutrientes del medio. El ámbito de estudio son zonas costeras abiertas, altamente dispersivas, donde el tiempo de residencia de los nutrientes en el agua es muy escaso, y donde se está concentrando la actividad de la industria acuícola en la última década. Los trabajos presentados en esta tesis surgen de dos proyectos de investigación cuyos objetivos coinciden en parte con los de esta tesis. El primero de ellos, el proyecto MEDVEG (2001-2004) del V Programa Marco de la UE, analizó el efecto de los vertidos de granjas marinas en praderas de *Posidonia oceánica* en el mediterráneo con el fin de obtener herramientas adecuadas para el monitoreo y gestión de estos ecosistemas. El segundo, un proyecto del Plan Nacional de Cultivos Marinos (JACUMAR, 2004-2007), tuvo como objetivo el desarrollo de herramientas que permitieran cuantificar el alcance espacial de los vertidos procedentes de las granjas marinas flotantes mediante el análisis de $\delta^{15}N$ en macrófitos.

Así, esta tesis se estructura en dos partes. En la primera parte se estudiaron las respuestas fisiológicas de las fanerógamas marinas *Posidonia oceanica* y *Cymodocea nodosa*, en praderas naturales expuestas a los vertidos procedentes de granjas marinas con el fin de evaluar las variables con mayor potencial como indicadoras de los aportes de nutrientes a estos ecosistemas (**Capítulos I y II**). En la segunda parte se desarrolló y aplicó un método para evaluar el alcance espacial de los aportes de la acuicultura en la columna de agua en ecosistemas costeros oligotróficos mediante el análisis del $\delta^{15}N$ en macrófitos (**Capítulos III, IV y V**).

En el **capítulo I** se evalúan una serie de descriptores a nivel fisiológico en la fanerógama marina *Posidonia oceánica* para detectar los efectos de aportes de la acuicultura en

este ecosistema. Las fanerógamas marinas son comunidades de amplia distribución en el mar mediterráneo y Canarias (i.e. en mares templados oligotróficos) y muy sensibles a impactos de origen antrópico, por tanto, variaciones en la fisiología de la planta podrían ser idóneos bioindicadores para evaluar el impacto de la acuicultura. Este estudio pone en evidencia el potencial de la señal isotópica del N, especialmente en el compartimento de epífitos, como bioindicador de la llegada de nitrógeno procedente de las granjas marinas a estos ecosistemas. En el **capítulo II** se profundiza sobre el efecto de los vertidos acuícolas en la variabilidad de la señal isotópica del N y del N total en los epífitos y en diferentes compartimentos de la fanerógama (hojas, rizomas y raíces). El contenido total de N (%N) en los tejidos de macrófitos bentónicos ha sido tradicionalmente utilizado como indicador de la disponibilidad de nutrientes en el medio. El objetivo de este capítulo es comparar la sensibilidad de ambos indicadores en diferentes compartimentos del ecosistema respecto a los cambios externos del régimen de nutrientes.

A pesar de que las algas y las praderas de angiospermas marinas se encuentran ampliamente distribuidas por todas las zonas costeras del mundo, su distribución a escala local es heterogénea y limitada por la profundidad. De esta forma su empleo como indicadores a lo largo de gradientes de nutrientes originados por la actividad antrópica (acuicultura en este caso) no es posible en algunos casos. Para tratar de solventar este problema, en el **capítulo III** de esta tesis se desarrolla una técnica basada en bioensayos con macrófitos bentónicos, aplicada por primera vez a los vertidos de granjas marinas en jaulas flotantes y situadas en zonas costeras abiertas y profundas, desprovistas de vegetación. En este capítulo se evalúan una serie de factores que pueden afectar a nuestra herramienta de análisis ($\delta^{15}N$ en macrófitos) tales como la especie de alga utilizada, la profundidad y el tiempo de incubación de los bioensayos. En el **capítulo IV** se aplica el método en base a los resultados del capítulo anterior y se aumenta el grado de resolución espacial para la caracterización de los gradientes de dispersión de los nutrientes desde las jaulas de cultivo. Por último, el **capítulo V** comprueba experimentalmente la eficacia del análisis de $\delta^{15}N$ en los tejidos de macroalgas cómo bioindicador del N procedente

de vertidos de la acuicultura. Para ello se evalúa la influencia del ratio de $\delta^{15}N$ y de la concentración de N del agua de mar y la influencia de factores ambientales, como la disponibilidad de luz, en el fraccionamiento isotópico de *Cystoseira mediterranea* con el fin de obtener información cerca de la capacidad de este macroalga de reflejar la señal isotópica del medio en el que se encuentra.

Listado de artículos publicados o enviados para su publicación

Capítulo I. Pérez, M., García-Sanz, M., Ruiz, J.M., Invers, O., 2008. Physiological responses of the seagrass *Posidonia oceanica* as indicators of fish farm impact. *Marine Pollution Bulletin* 56, 869-879.
Factor de impacto: 2.562

Capítulo II. García-Sanz, M., Ruiz, J.M., Ruiz, M., Pérez, M., *in prep* Assessing the response of total N and $\delta^{15}N$ in different compartments of seagrasses exposed to fish-farm wastes. *Botanica Marina.*
Factor de impacto: 0.767

Capítulo III. García-Sanz, M., Ruiz, J.M., Ruiz, M., Pérez, M., González, M.N., García, R., *in press*. An evaluation of a macroalgal bioassay tool for assessing the spatial extent of nutrient release from offshore fish farms. *Marine Environmental Research.*
Factor de impacto: 2.032

Capítulo IV. García-Sanz, M., Ruiz, J.M., Ruiz, M., Pérez, M., *in prep*. Assessment of offshore fish-farm waste dispersal using nitrogen stable isotope ratio ($\delta^{15}N$) in macroalgal bioassays. *Estuarine Coastal and Shelf Science.*
Factor de impacto: 2.072

Capítulo V. García-Sanz, M., Pérez, M., *in prep*. Experimental evidence supports the use of $\delta^{15}N$ in macroalgal tissues as an indicator of available N sources. *Journal of Experimental Marine Biology and Ecology.*
Factor de impacto: 2.074

Barcelona, 11 de Diciembre, 2009

Los directores de la presente tesis, Marta Pérez Vallmitjana y Juan Manuel Ruiz Fernández, certifican que María García Sanz ha participado activamente en el desarrollo del trabajo de investigación asociado a cada uno de los artículos presentados en esta Tesis Doctoral, así como en su elaboración. En concreto, su participación en cada una de las tareas ha sido la siguiente:

- Planteamiento de los objetivos de cada uno de los trabajos
- Planificación y ejecución de los experimentos de campo y laboratorio
- Procesado y análisis de las muestras obtenidas
- Análisis de los datos
- Redacción de los artículos y seguimiento del proceso de revisión de los mismos.

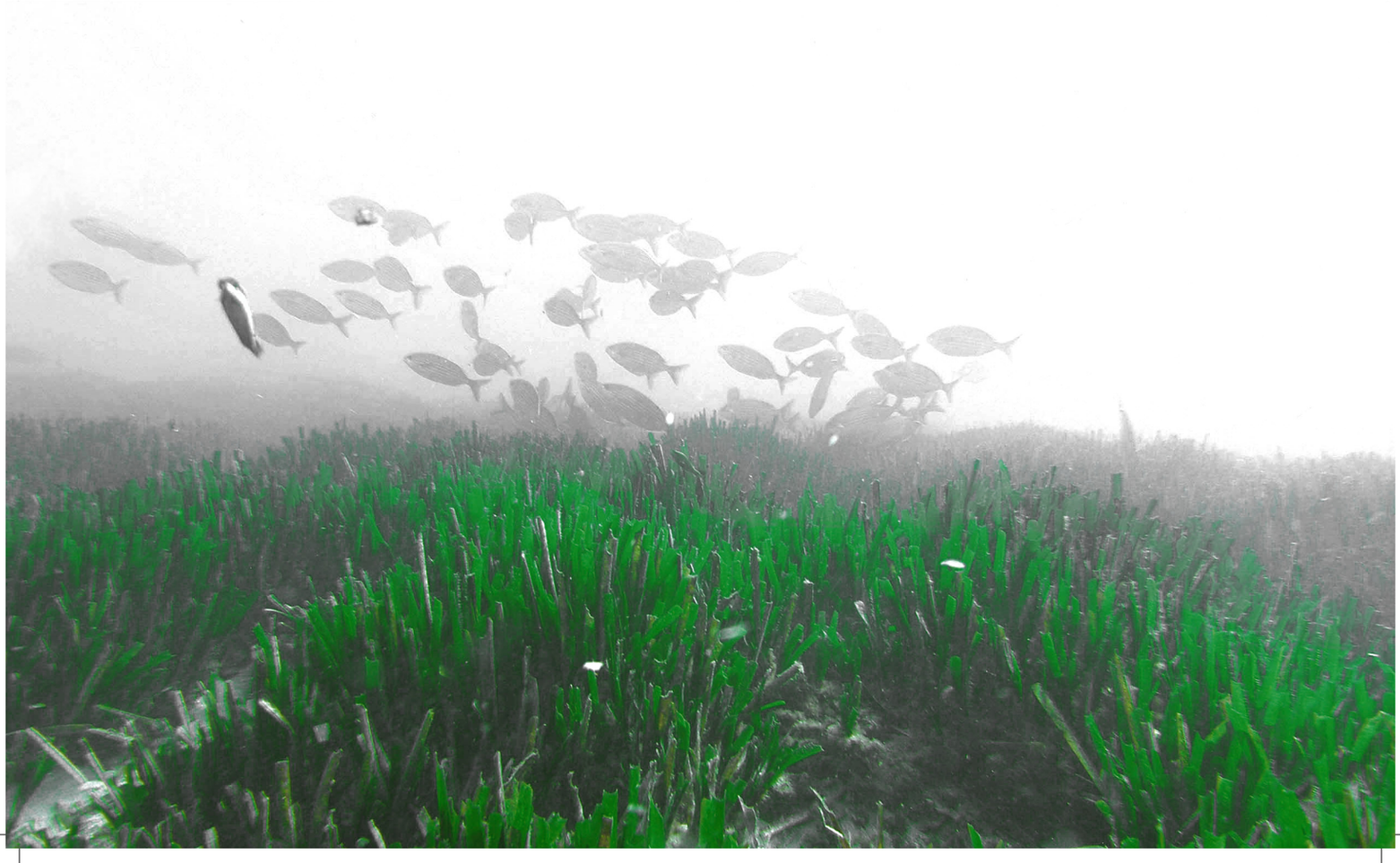
Finalmente, certifican que ninguno de los coautores de los artículos presentados anteriormente y que forman parte de la Tesis Doctoral de María García Sanz ha utilizado o tiene previsto utilizar implícita o explícitamente estos trabajos para la elaboración de otra Tesis Doctoral.

Fdo:

Marta Pérez Vallmitjana Juan Manuel Ruiz Fernández

Chapter 1

Physiological responses of the seagrass *Posidonia oceanica* as indicators of fish-farm impact



Respuestas fisiológicas de la fanerógama marina *Posidonia oceanica* como indicadoras del impacto de granjas marinas

Resumen

El desarrollo de la acuicultura a lo largo de la costa del mar Mediterráneo degrada el medio marino, en particular las praderas de *Posidonia oceanica*, las cuales, en casos extremos, muestran una alta mortalidad. En este artículo estudiamos los efectos de la entrada de materia orgánica y de nutrientes derivados de los vertidos de tres granjas marinas, localizadas a lo largo de la costa mediterránea, en la fisiología de *P. oceanica*. Para ello medimos variables fisiológicas tales como el contenido total de nitrógeno (N), la concentración y composición de aminoácidos libres (FAA), los ratios del isótopo estable del nitrógeno ($\delta^{15}N$), el contenido total de fósforo (P) y el contenido total de carbohidratos no estructurales (TNC) en los tejidos y epífitos de plantas afectadas por las descargas orgánicas (estaciones altamente impactadas: HI, y estaciones ligeramente impactadas : LI) y comparamos estos resultados con aquellos obtenidos en sitios de referencia (estaciones control: C). Los valores obtenidos en las inmediaciones de las granjas en todos los descriptores analizados en los epífitos de *P. oceanica* fueron en general, mucho mayores que aquellos obtenidos en los sitios control. Las hojas no respondieron consistentemente en ningún caso. El contenido total de N y el δN^{15} en epífitos, junto con el contenido total de P en rizomas y epífitos fueron los descriptores fisiológicos que mostraron una respuesta más consistente a los vertidos de granjas marinas. En base a nuestras observaciones podemos afirmar que las actividades de las granjas marinas afectan severamente a los parámetros fisiológicos de las praderas de *P. oceanica* cercanas a las instalaciones de granjas marinas. Por lo tanto, podemos concluir que los cambios en estos parámetros fisiológicos podrían ser buenos indicadores de la degradación ambiental marina en estudios que monitoricen los efectos de los vertidos procedentes de la acuicultura.

Abstract

The development of aquaculture along the Mediterranean coastline degrades the marine environment, in particular *Posidonia oceanica* meadows, which, in extreme cases, show high mortality. Here we studied the effects of organic matter and nutrient input from the effluents of three fish farms, located along the Mediterranean coast, on *P. oceanica* physiology. For this purpose, we measured physiological variables such as total nitrogen (N) content, free amino acid (FAA) concentration and composition, N stable isotope ratio ($\delta^{15}N$), total phosphorus (P) content and total non-structural carbohydrate (TNC) content in plant tissues and epiphytes affected by organic discharges (highly impacted stations: HI, and less impacted stations: LI) and compared these results with those obtained in references sites (control stations: C). For all the descriptors analyzed in *P. oceanica* epiphytes, the values recorded in the vicinity of cages were, in general, much higher than those in C. Leaves did not respond consistently in any case. Total N content and δN^{15} in epiphytes together with the total P content in rhizomes and epiphytes were the physiological descriptors that showed the most consistent responses to fish-farm effluents. On the basis of these observations, we conclude that fish-farm activities strongly affect the physiological parameters of nearby *P. oceanica* meadows. We propose that changes in these physiological parameters may be useful indicators of marine environmental degradation in studies that monitor the effects of fish farming.

Keywords: *Posidonia oceanica*; Mediterranean Sea; Fish farm; Bioindicators; Physiology; Epiphytes.

Introduction

Aquaculture is an expanding industry in many parts of the world and currently accounts for almost 50 percent of the world's fish production. It has been predicted that by 2030 aquaculture production will have to increase by at least 40 million tons in order to cover the global demand for seafood. A contrasting scenario is that faced by most wild fisheries, which are at their maximum level of sustainable exploitation (UNEP, 2002; Subasinghe, 2004; FAO, 2006). Marine aquaculture developed in floating cages in open water accounts for a significant part of total aquaculture production. In particular, in the Mediterranean Sea, this activity grew exponentially throughout the 90s (Basurco and Lovatelli, 2003). This rapid expansion, now somewhat stabilized, makes this industry a potential environmental risk in marine coastal ecosystems because of the nutrients and organic matter released into the environment. Consequently, methods and strategies to evaluate the effects of the fish farm waste on the ecosystem are required (Chua, 1992; Wu et al., 1995; Karakassis, 2001; Pergent-Martini et al., 2006).

Several studies have reported that the effluents (uneaten fish food, fecal pellets) from fish farms affect the environmental conditions of the water column and the sediment around the cages, especially in intensive cultures of carnivorous fish, which require large amounts of manufactured feed (Wu et al., 1994, Dalsgaard and Krause-Jensen, 2006, Holmer et al., 2007).

In the water column, fish farm effluents can stimulate phytoplankton and bacterial development and hence reduce column transparency (Guo and Li, 2003; Sakami et al., 2003). However, most surveys report little deterioration of water column characteristics in sites with high water circulation and vigorous tidal flushing (Nordvarg and Johansson, 2002; Soto and Norambuena, 2004; Sarà, 2007). In the Mediterranean Sea, the water quality around fish farms appears to be relatively unaffected, showing localized or no effects of dissolved wastes on most of the variables studied to date (Pitta et al., 1999; La Rosa et al., 2002; Maldonado et al., 2005).

This lack of effect can be attributed to fast dilution (Pitta et al., 2006) and to the rapid transition of nutrients through the food web from phytoplankton upwards (Machias et al., 2004, 2005). At large spatial and temporal scales, it has been reported that the effects of fish farms on water quality are not relevant (Karakassis et al., 2005; Pitta et al., 2005).

In contrast, the impact of fish farms on benthic ecosystems are far more severe, resulting in organic enrichment of sediments (Karakassis et al., 1998; Sarà et al., 2004; Holmer et al., 2005), which enhances bacterial activity (La Rosa et al., 2001; Vezzuli et al., 2002) and, in particular anaerobic activity, which leads to reduced sediments (Holmer and Kristensen, 1992; Holmer and Frederiksen, 2007). A decrease in diversity has also been found in meiofauna communities (Mazzola et al., 2000; Mirto et al., 2002), but no negative effects have been detected in macrofauna communities associated with *Posidonia oceanica* meadows (Apostolaki et al., 2007).

The effects of fish farms on benthic primary producers, especially seagrass, are also widely reported (see reviews in Holmer et al., 2003 and Pergent-Martini et al., 2006). The shade cast by the cages used in aquaculture and the accumulation of organic waste on the seabed significantly reduces the density of *P. oceanica* shoots to such an extent that they are absent in the vicinity of these cages (Dimech et al., 2000; Ruiz and Romero, 2001; Cancemi et al., 2003). Furthermore, the organic enrichment around the cages can increase epiphytic growth and herbivore pressure (Delgado et al., 1997; Pergent et al., 1999), thereby limiting photosynthesis of seagrass and hence reducing non structural-carbohydrate pools (Ruiz et al., 2001). Finally, fish farm effluents can lead to an increase in nutrient availability in the sediment porewater and to elevated sulfide concentrations in the root zone, which are among the main factors that negatively affect seagrass health and survival (Pérez et al., 2007).

The release of nutrients from fish farms has traditionally been monitored by measuring physical or chemical variables. However, this approach provides little information about

environmental quality (Lyngby, 1990) because of the spatial and temporal variability associated with these measures (Wolanski et al., 2000; Karakassis et al., 2001). In addition, changes in physical and chemical variables are detectable only close to the discharge point (Samocha and Lawrence 1997), while the biological influence of effluents can extend much further (Jones et al., 2001). Biological indicators integrate short-lived nutrient pulses and provide information about the nutrient source, and the bioavailability of the nutrients. In this regard, the present tendency is to apply assessment and environmental monitoring methods of coastal systems that combine traditional techniques of water quality analysis (physical-chemical variables) with newly developed biological indicators (Costanzo et al., 2001; Dalsgaard and Krause-Jensen, 2006).

Marine macrophytes can provide information about the ecological impact of anthropogenic nutrient inputs (Udy and Dennison, 1997; Lyngby et al., 1999; Cole et al., 2005). Studies on seagrass response to specific impacts describe a wide range of variables to assess plant health status, and several can be used as bioindicators of fish farm impact. For example, reductions in distribution area, cover and density, decrease in shoot size, appearance of necrosis in leaves (Dimech et al., 2000; Cancemi et al., 2003), reductions in vertical rhizome growth (Marbà et al., 2006) and changes in epiphyte load (Frankovich and Fourqurean, 1997) have been tested as indicators of stress in *P. oceanica* meadows and used in monitoring programs. Plants exposed to high nutrient loads also show physiological changes, such as increases in tissue nutrient content, alterations in free amino acid content and composition, decreases in carbon reserves, and variations in the nitrogen stable isotope ratio and trace metal content, among others (Udy and Dennison, 1997; Pergent-Martini, 1999; Invers et al., 2004; Vizzini and Mazzola, 2004; Pérez et al., 2007).

Here we examined the physiological response of *P. oceanica* to an increase in nutrient availability as a result of fish farm effluents. For this purpose, several physiological descriptors were measured in *P. oceanica* meadows at a range of distances from fish farm facilities in three

localities in the Mediterranean Sea (Italy, Greece and Spain). The descriptors were: total nitrogen (N) content, free amino acid (FAA) concentration and composition, N stable isotope ratio ($\delta^{15}N$), total phosphorus (P) content and total non-structural carbohydrate (TNC) content in epiphytes and seagrass tissues. Here we report on the capacity of each descriptor to respond to nutrient inputs from fish cages and evaluate their usefulness as early indicators of marine environmental degradation caused by aquaculture activities.

Materials and Methods

Study sites and sampling design

The study was conducted in three fish farms located in Greece, Italy and Spain (Figure 1). All the farms are situated in exposed areas with high water exchange and all have a relatively deep bottom (from 18 to 27 m, Table 1). The main species cultured were gilthead bream *(Sparus aurata*) and sea bass (*Dicentrachus labrax*), which were fed manually with dry pellets. In all cases, the seabed under the cages presented dead *P. oceanica* rhizomes, which indicated the presence of a meadow before fish farm activity began, as confirmed by local researchers (Karakassis and Tsapakis, pers. com.; Sánchez-Lizaso, pers. com.).

Sampling campaigns were conducted in September 2002 in Italy, in June 2003 in Greece and in September 2003 in Spain, coinciding with the seasons of maximum activity in the farms and therefore high waste release. In each fish farm, three sampling stations distributed along a transect in a downstream direction were selected in areas covered by *P. oceanica* but at a range of distances from the source of farm effluent: i) the sampling station with highest influence (HI) of effluents was in the meadow closest to the farm (5-15 m from net cages); ii) a less affected

station (LI) was placed further from the farm (30-40 m from net cages); and iii) the control station (C) was located in an area far enough (around 1000 m) from the farm to be considered not influenced by effluents (Ruiz et al., 2001). The depth of the sampling stations was kept constant or within a narrow range at each sampling site.

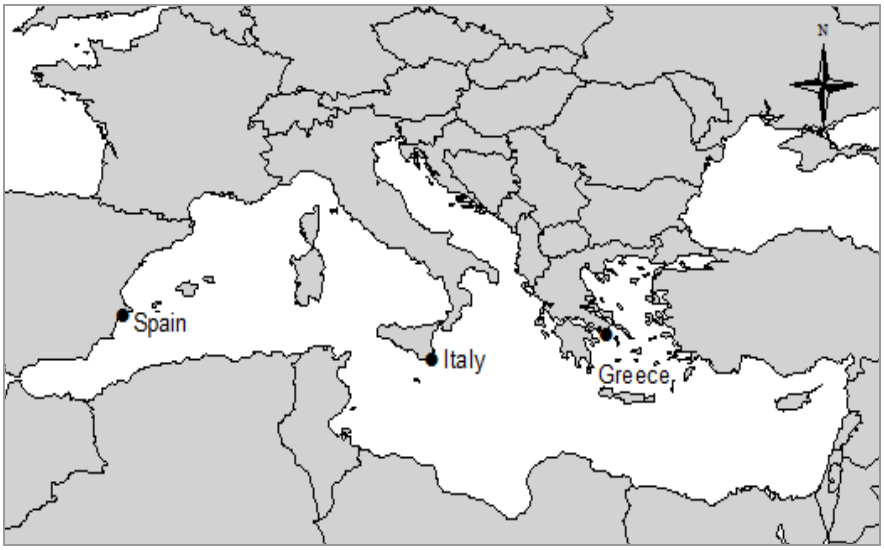


Figure 1. Location of the three fish farms in the Mediterranean.

Sampling and plant processing

At each selected station, SCUBA divers collected three samples with a 17-cm diameter core from *P. oceanica* patches. For each sample, we separated shoots, live rhizomes and live roots. The leaves of each shoot were separated and only young leaves (0-50 days old) were kept for analysis. Epiphytes were gently removed from the leaves with a razor blade and kept for analyses. Epiphytes and the rest of the plant material (young leaves, rhizomes and roots) were dried at 70ºC until constant weight (24-48 h) and ground to a fine powder using an agate mortar

and pestle. Dry samples were preserved in a desiccator at room temperature until analyses for total N and P content (in young leaves, epiphytes, rhizomes and roots), $\delta^{15}N$ (in young leaves and epiphytes) and TNC (in rhizomes and roots). A subsample of rhizomes and roots were kept frozen (-70ºC) for FAA analysis.

Table 1. Site and characteristics of the three fish farms.

Site	Location	Start of operation	Depth (m)	Fish prod. ($t\ y^{-1}$)	Nº of net cages
Italy	Pachino bay, Sicily	1992	23-34	1150	24
Greece	Cape Sounion	1996	15-20	400	20
Spain	El Campello, Alicante	1996	26-28	260	24

Production is given as tons of fresh weight per year.

Analytical procedures

Total N content (% DW) in epiphytes, young leaves, rhizomes and roots was measured using a Carlo-Erba CNH elemental autoanalyzer.

For FAA determination, frozen tissues from rhizomes and roots were ground in 20 ml of 40.05N HCl and then centrifuged for 5 min at 10,000 rpm. The supernatant was filtered in a microcentrifuge using low-binding regenerated cellulose Millipore ultra-free filters to exclude peptides with molecular masses greater than 10kDa. FAA content was measured using an amino acid autoanalyzer via ionic exchange chromatography, following the method of Spackman et al. (1958).

For $\delta^{15}N$ analysis, pulverized young leaves and epiphytes were encapsulated. $\delta^{15}N$ was determined using a Finnigan Mat Series IRMS analyzer in continuous flow configuration with an analytical error always less than 0.2 ‰. Isotopic data were reported in common delta (δ) units referred to atmospheric nitrogen standards (N_2) using the following equation (Peterson and Fry, 1987):

$$\delta^{15}N\ (‰) = (R_{sample} / R_{standard}) - 1) \times 10^3$$

where, $R = {}^{15}N / {}^{14}N$

Total P content (% DW) in epiphytes, young leaves, rhizomes and roots was determined by a dry-oxidation acid hydrolysis extraction followed by a colorimetric analysis of phosphate concentration of the extract (Fourqurean et al., 1992).

TNC content was measured in rhizomes and roots following the method described in Alcoverro et al. (1999), based on Yemm and Williams (1954). Sugars (sucrose and hexoses) were solubilized from dry and ground tissues by four sequential extractions in 95% (v/v) ethanol at 80 ºC for 15 min. The ethanol extracts were evaporated under a stream of air at room temperature and the residues dissolved in deionized water for analysis. Starch was extracted from the ethanol-insoluble residue by keeping it for 24 h in 1N NaOH. Sugar and starch contents of extracts were determined spectrophotometrically using a resorcinol and anthrone assay, respectively, with sucrose as standard. Results are expressed in % of dry weight of sucrose and starch.

Statistical analysis

For each variable, three replicates were analyzed and means and standard errors were calculated. The significance of differences in *P. oceanica* tissues and epiphytes between stations

(factor 'zone') was tested using one-way analysis of variance (ANOVA) for total N and P content in epiphytes, young leaves, rhizomes and roots; for TNC content and FAA concentration in rhizomes and roots; and for the $\delta^{15}N$ in young leaves and epiphytes.

Results

Total nitrogen (N) content

Total N content in the plant tissues and in epiphytes showed a significant increase towards the cages in all the sites and fractions analyzed except in young leaves collected from farms in Italy and Spain and rhizomes and roots from Italy (Figure 2 and Table 2).

Epiphyte N content showed a significant increase in all the sites, although the highest effect was in Italy (the largest fish farm), which registered a 3-fold increase above the C value. The lowest value for this parameter was found in Spain (the deepest fish farm), which showed a 1.2-fold increase above the value recorded in C. Among the diverse tissues analyzed, the greatest response to effluents from fish farms was for rhizomes, which showed a significant increase in N content, with between 2- and 3.5-fold higher values near the farm compared to C sites in Spain and Greece respectively. Roots had the lowest N content of all the fractions and were between 1.2- and 1.5-fold higher than that recorded in C in Greece and Spain. Young leaves showed a significant response (1.3 fold increase) only close to the fish farm in Greece.

Table 2 . Summary of ANOVA results for Italy, Greece and Spain campaigns.

Variable	Tissue	Italy		Greece		Spain	
		% variance	p	% variance	p	% variance	p
Total N	Young leaf	---	NS	87.0	**	---	NS
	Rhizomes	---	NS	81.2	**	73.8	**
	Roots	---	NS	77.3	*	58.6	*
	Epiphytes	97.3	***	85.4	**	70.0	*
FAA	Rhizomes	---	NS	92.0	***	77.0	*
	Roots	91.4	*	67.8	*	85.62	**
$\delta^{15}N$	Young leaf	---	NS	---	NS	---	--
	Epiphytes	94.8	***	92.9	***	---	--
Total P	Young leaf	---	NS	74.1	*	55.7	*
	Rhizomes	74.4	***	96.8	***	77.5	**
	Roots	---	NS	79.7	**	61.2	*
	Epiphytes	94.8	***	88.4	***	97.8	***
TNC	Rhizomes	71.1	*	73.4	*	52.8	*
	Roots	---	NS	81.4	**	91.3	***

For the sake of simplicity, only the percentage of variance caused by each variable and the test level of significance are shown. H_o: there are no significant effects between "zones" (i.e. between HI, LI and C stations). NS = not significant, *p < 0.05; **p< 0.01; ***p < 0.001. % variance corresponding to non-significant results must be considered due to random.

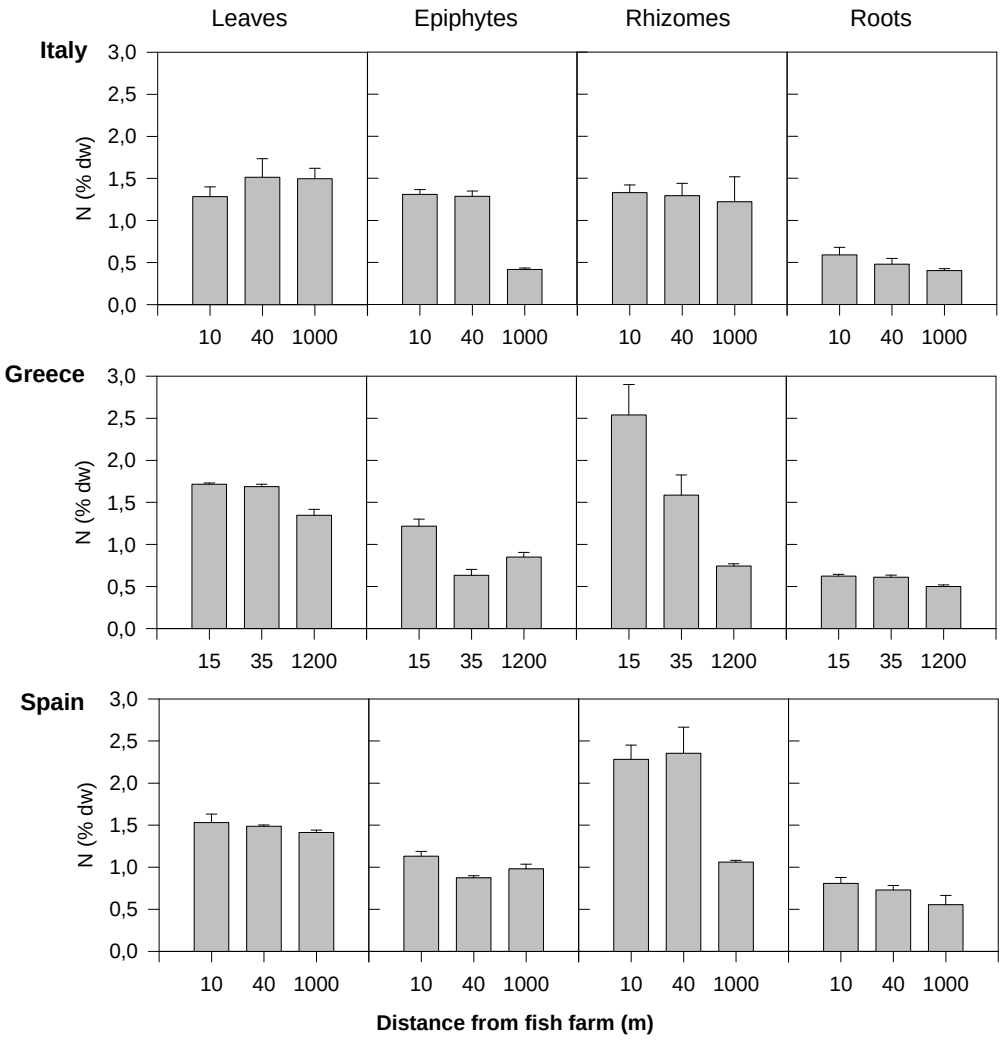


Figure 2. Total nitrogen content in *P. oceanica* tissues and epiphytes from vegetated seabed in the proximity of fish farms in Italy, Greece and Spain, with depths of 22 m, 16 m and 28 m depth, respectively. Stations along the downstream transect: HI, highly impacted station (10-15 m); LI, less impacted station (35-40 m), and C, control station (1000-1200 m). Vertical bars correspond to the standard error of the mean.

Free amino acid concentration (FAA)

Total FAA concentration in rhizomes and roots from the three sites showed a significant increase with proximity to the fish farm, except in rhizomes from Italy (Figure 3 and Table 2). Total FAA in rhizomes from Greece and Spain were 1.7- and 2.6- fold higher in HI stations compared to C respectively. FAA concentration increased in roots, which showed values 2.2- and 3-fold higher in Greece and Spain respectively.

The rise in FAA concentration in rhizomes in HI stations compared to C in fish farms in Greece and Spain was due mainly to an increase in asparagine and arginine, which accounted for between from 8 and 70% and between 16 and 25% of the total FAA increase respectively.

The increase in FAA concentration in roots sampled from Italy and Spain was explained mainly by the increase in asparagine, serine and alanine, which accounted for between 35 and 40%, 8 and 30% and 13 and 20% of the total FAA increase respectively. In addition, in Italy, roots from the HI station presented a 4-fold increase in *g*-aminobutyric acid (GABA) over that recorded in C. In Greece, despite the significant increase in total FAA concentration in roots, variability among replicates caused a non-significant specific FAA increase at the HI station compared to LI station.

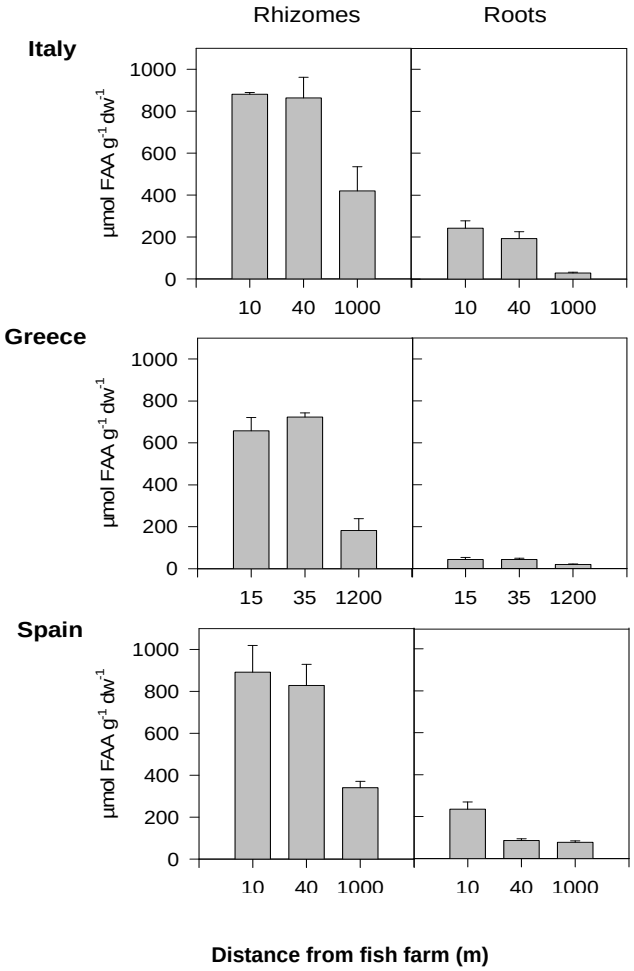


Figure 3. Total free amino acid concentration in P. oceanica rhizomes and roots from vegetated seabed in the proximity of the fish farms in Italy, Greece and Spain, with depths of 22 m, 16 m and 28 m, respectively. Stations along the downstream transect: HI, highly impacted station (10-15 m); LI, less impacted station (35-40 m), and C, control station (1000-1200 m). Vertical bars correspond to the standard error of the mean.

Nitrogen Stable Isotope Ratio ($\delta^{15}N$)

The $\delta^{15}N$ of the epiphytes was significantly higher in the stations closest to the fish farms than in C (Figure 4 and Table 2) in Italy and Greece. The effect was more pronounced in Italy, with a 3-fold increase in the HI station compared to C whereas in Greece the increase between these two stations was about 1.7-fold. In contrast, no significant differences ($p > 0.05$) among stations were found in young leaves at any site, although $\delta^{15}N$ tended to decrease along the transect.

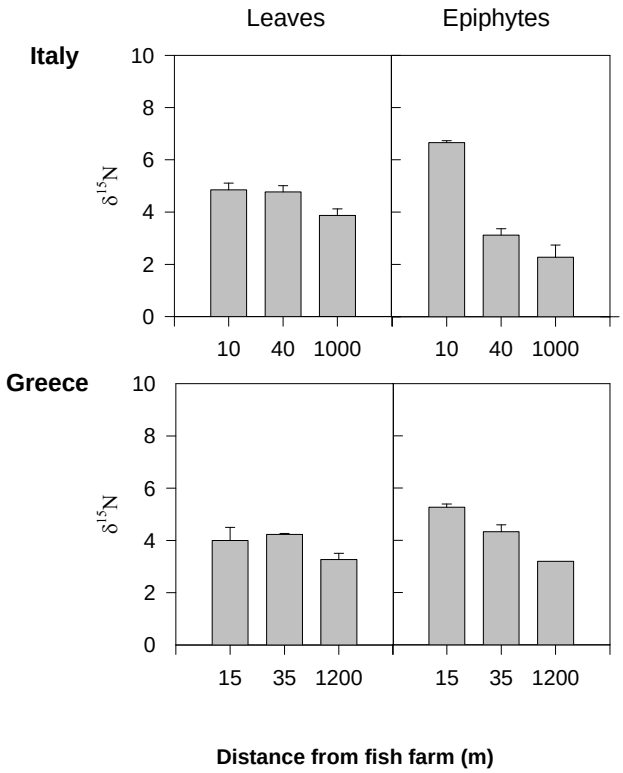


Figure 4. Nitrogen isotopic ratio ($\delta^{15}N$) in *P. oceanica* leaves and epiphytes from vegetated seabed in the proximity of the fish farms in Italy and Greece, with depths of 22 m and 16 m, respectively. Stations along the downstream transect: HI, highly impacted station (10-15 m); LI, less impacted station (35-40 m), and C, control station (1000-1200 m). Vertical bars correspond to the standard error of the mean.

Total phosphorus (P) content

Total P content in epiphytes and plant tissues showed a significant increase in the HI stations in all sites, except for young leaves and roots in Italy (Figure 5 and Table 2). Epiphytes showed the most significant response in all cases, with increases of 2- to 5.5-fold followed by rhizomes, in which total P content increased from 2.5- to 5-fold in HI stations compared with C. Total P content in young leaves increased 1.1- and 1.8-fold in Spain and Greece respectively and in roots 1.5- and 1.6-fold in Greece and Spain respectively compared with C values.

Total non-structural carbohydrate (TNC)

The TNC content of rhizomes and roots from the three sites showed a clear and similar pattern, with a significant decrease towards fish farms, with the exception of roots in Italy (Figure 6 and Table 2). The TNC content in rhizomes was from 1.3- to 1.8-fold lower in the HI stations compared with C. A 1.4- and 2.7-fold decrease in TNC content of roots was recorded in Spain and Greece respectively respect to the C values.

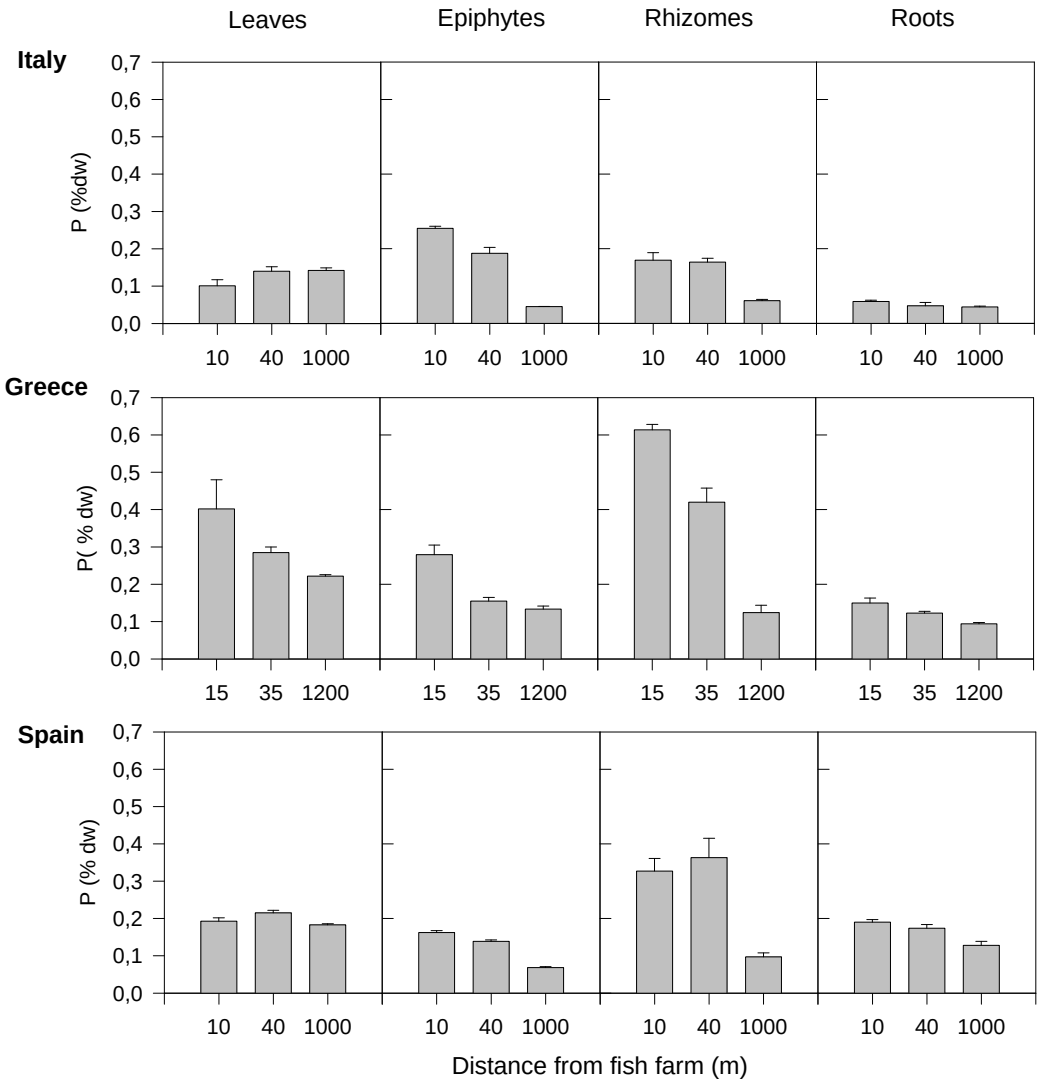


Figure 5. Total phosphorus content in *P. oceanica* tissues and epiphytes from vegetated seabed in the proximity of the fish farms in Italy, Greece and Spain, with depths of 22 m, 16 m and 28 m, respectively. Stations along the downstream transect: HI, highly impacted station (10-15 m); LI, less impacted station (35-40 m), and C, control station (1000-1200 m). Vertical bars correspond to the standard error of the mean.

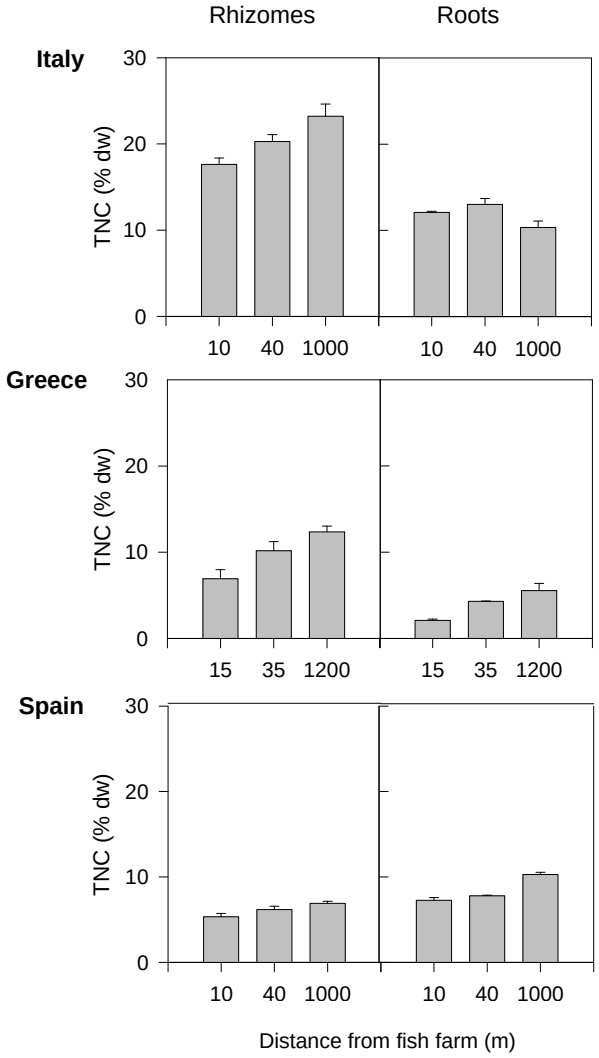


Figure 6. Total non-structural carbohydrates content (TNC) in *P. oceanica* rhizomes and roots from vegetated seabed in the proximity of the fish farms in Italy, Greece and Spain, with depths of 22 m, 16 m and 28 m, respectively. Stations along the downstream transect: HI, highly impacted station (10-15 m); LI, less impacted station (35-40 m), and C, control station (1000-1200 m). Vertical bars correspond to the standard error of the mean.

Discussion

Our results indicate that the changes in the physiological variables studied in *P. oceanica* and its epiphytes in the proximity of aquaculture facilities are caused by the effluents. We conclude that these variables can be used to detect environmental degradation produced by fish farms.

Although epiphyte load in seagrasses is related to nutrient availability along natural or experimental nutrient gradients (Borum, 1985; Tomasko and Lapointe, 1991; Frankovich and Fourqurean, 1997; Peterson et al., 2007) other factors such as top-down control of epiphyte abundance by grazers can mask this relationship (Neckles et al., 1993; Lin et al., 1996; Heck et al., 2000; Prado et al., 2007). However, the control of epiphyte abundance by other environmental (e.g. depth) and biological (e.g. grazers) factors is complex (Ruiz et al., 2001). Therefore, epiphyte abundance does not appear to be a good indicator of changes in nutrient regime. In contrast, epiphyte nutrient content showed a close relationship with external nutrient loads, as indicated by the response of all the physiological variables we measured in the epiphytes of *P. oceanica* (N content, $\delta^{15}N$ and P content). In all sampling sites in HI stations, these descriptors increased, indicating that effluents from these facilities enhanced nutrient assimilation by this seagrass.

Furthermore, nutrient content in seagrass tissues also increases along natural nutrient gradients (Duarte, 1990; Fourqurean et al., 1992; Lee et al., 2004, Romero et al., 2006) and after experimental nutrient additions (Short et al., 1995, Udy and Dennison 1997, Moore and Wetzel, 2000, Invers et al., 2004, Perez et al., 2007). These studies describe several morphological and physiological changes of seagrass in response to altered environmental conditions. In general, physiological responses were reported to be better indicators of saturating nutrient supply to an environment than morphological changes.

An increase in N availability generally leads to a rise in N uptake (Pérez-Llorens and Niell, 1995; Pedersen et al., 1997) because seagrasses are unable to down-regulate the uptake of this element (Touchette and Burkholder, 2000). In our study, increases in N uptake was reflected by a higher N content in all tissues collected in the proximity of fish farms, except in Italy. The highest response was found in rhizomes. This observation is consistent with the storage capacity of this organ (Invers et al., 2004). In leaves, this pattern was less consistent, and a relation between N content and nutrient availability was found only in Greece. These results coincide with those reported by Lee et al., (2004), who studied *Zostera marina.* These authors described increases in N content in leaves exposed to nutrient enrichment but they found that natural variability in this parameter was too high for it to be considered as a good indicator.

The lack of response of total N content in *P. oceanica* tissues collected in Italy could be attributed to the effect of sediment conditions on N metabolism (Pérez et al., 2007). This aquaculture facility was the largest in this study and showed the highest rate of sedimentation under cages (Holmer et al., 2007). At this fish farm, sediment has the highest sulphate reduction rate together with the highest levels of acid-volatile fraction of the sulfide pools among sampling sites (Holmer and Frederiksen, 2007). These sediment characteristics, related to low oxygen concentrations, may inhibit N assimilation and protein synthesis, as described for *Z. marina* (Pregnall et al., 1984; Smith et al., 1988) and *P. oceanica* (Pérez et al., 2007).

An increase in total N content generally implies an increase in FAA content (Udy and Dennison, 1997; Invers et al., 2004). N taken up above the requirements for growth is quickly assimilated and stored as amino acids because of the toxicity of ammonium (Touchette and Burkholder, 2000). This observation is confirmed by our results. The response of FAA concentration in *P. oceanica* rhizomes and roots along the study transects was significantly higher in HI stations in Greece and Spain. However, this observation was not found in rhizomes

collected in Italy. This finding could be explained by the lack of enhanced N uptake at this fish farm.

Changes in FAA composition have also been related to nutrient enrichment and sediment conditions (Udy and Dennison, 1997; Pérez et al., 2007). Increases in FAA content in rhizomes in the proximity of fish farms are due mainly to an increase in asparagine and arginine content. The former has been related to N storage (Udy and Dennison, 1997; Kohl et al., 1998), especially in conditions of N excess and under stress, in which protein synthesis would be impaired, such as occurs under low oxygen conditions in the sediment (Pérez et al., 2007). In *P. oceanica* roots collected in Italy and Spain, the increase in FAA concentration was caused mainly by asparagine, serine and alanine. The accumulation of asparagine, as explained above, is a response to higher N availability; however, the presence of serine and alanine may be related to partial anaerobic metabolism in the root/rhizome system (Kohl et al., 1998). Moreover, in Italy, the increase in GABA content in roots would corroborate stronger hypoxia conditions in the sediment since the formation of this amino acid from others, like glutamine and glutamic acid, has been described during periods of anaerobic conditions (Pregnall et al., 1984; Smith et al., 1988; Pérez et al., 2007).

$\delta^{15}N$ has recently been used to trace the fluxes of aquaculture wastes in near shore waters (Vizzini and Mazzola, 2004, 2006; Lojen et al., 2005; Dalsgaard and Krause-Jensen, 2006, Holmer et al., 2007). $\delta^{15}N$ is an extremely powerful and relatively simple parameter with which to monitor the fate of fish farm-derived organic matter in coastal marine systems, and a sensitive indicator of the spatial influence of cage-derived N (Costanzo et al., 2001; Jones et al., 2001). Plants exposed to fish farm effluents show significantly higher $\delta^{15}N$ signatures than those collected at reference sites. This shift seems to be caused mainly by the higher $\delta^{15}N$ of the N-rich fish wastes rather than by changes in physiological discrimination during N acquisition (Yamamuro et al., 2003; Cohen and Fong, 2005) or by other factors such as light intensity or differences in species (Grice et al., 1996). This hypothesis is corroborated by our results, which

show that $\delta^{15}N$ in epiphytes declined with distance from fish farms at all the sites. On the basis of this observation, we propose that $\delta^{15}N$ would be useful to trace the fate of waste from aquaculture facilities.

P content in tissues and epiphytes of seagrasses also increases following nutrient gradients and experimental additions (Perez et al., 1991; Fourqurean et al., 1992; Udy and Dennison 1997; Romero et al., 2006). In general, our results are consistent with this trend since total P content in *P. oceanica* epiphytes and below-ground tissues increased with proximity to fish cages in all the sites except in leaf and root tissues sampled in Italy. The lack of response in leaves from Italy can be attributable to factors such as a loss of P uptake capacity as a result of leaf loss by herbivory or competition for this resource with epiphytes. Moreover, as in the case of N uptake in the seagrass samples collected in Italy, the P uptake can also be affected by sediment conditions (Pérez et al., 2007), which could also explain the lack of response observed in leaves and roots. The lower P content in Italy in relation with the other two sites supports this hypothesis. Furthermore, rhizomes showed a consistent response to the proximity of fish farms, probably because this organ is where P is immobilized.

Decreases in carbohydrate reserves after nutrient increases have been repeatedly observed (Delgado et al., 1999; Lee and Dunton, 1999; Alcoverro et al., 2001; Invers et al., 2004). Decreases in this variable have recently been used as a bioindicator of human pressure (Romero et al., 2007). In our study, the lower TNC content in rhizomes sampled close to fish cages in Italy, Greece and Spain, and in roots in Greece and Spain can be attributed to: (a) higher utilization of carbon skeletons for N assimilation (Invers et al., 2004) in those cases in which total N content increases close to the cages (decreases in carbon reserves fit with increases in FAA); (b) lower synthesis of photosynthates caused by lower light availability as a result of a reduction in water column transparency and/or the shadowing by epiphyte overgrowth (Ruiz and Romero, 2001); (c) a reduction of photosynthetic biomass as a result of high herbivore pressure in Italy and Greece (Holmer et al., in press), which may promote the use

of existing carbon reserves to maintain respiration of below-ground tissues (Alcoverro et al., 2001); and; (d) the interference of oxygen shortage at the sediment with carbon metabolism of the plant, which reduces the transport of photosynthates from leaves to below-ground tissues and causes the over-consumption of carbohydrate reserves to maintain rhizome/root anaerobic metabolism. This is much less efficient in energy acquisition than the aerobic mechanism (Zimmerman and Alberte, 1996; Liao and Lin, 2001; Perez et al., 2007). The relative importance of each of these four causes of TNC reduction varied depending on the sampling site.

In summary, among the physiological indicators measured in *P. oceanica*, total N content and δN^{15} in epiphytes showed the most consistent responses to fish farm proximity together with the total P content in rhizomes and epiphytes. Total TNC content also showed a robust response, especially in rhizomes, the main storage organ in *P. oceanica*. Finally, the specific composition of the FAA pool has been found to be as sensitive measure to assess the stress conditions that affect *P. oceanica*, such as increased N availability or oxygen shortage in the sediment. The other physiological changes detected in *P. oceanica* tissues showed a general response to fish farm inputs but may be affected by interferences caused by other nutrient-induced effects such as anoxia, so their response is not so clear.

Although several authors have reported responses to nutrient enrichment in N content in leaves (Touchette et al., 2003; Invers et al., 2004; Leoni et al., 2007), our results indicate that rhizomes are the most robust compartment in the plant to measure changes associated with nutrient inputs. Epiphytes are also robust and reliable indicators of moderate nutrient enrichment derived from fish farms.

Our results confirm the usefulness and reliability of seagrass physiological descriptors to identify environmental degradation in relation to classic water column and sediment variables. Furthermore, the intensity of the response shown by some of these descriptors in

seagrass indicates that they respond to fish farm effluents even before changes in community structure occur (plant biomass, shoot density, meadow cover, etc.). Given the sensitivity of these descriptors to these organic inputs, we conclude that they could be used as monitoring tools in aquaculture management.

Chapter **2**

Assessing the response of total N and $\delta^{15}N$ in different compartments of seagrasses exposed to fish-farm wastes

Evaluación de la respuesta de N total y $\delta^{15}N$ en diferentes compartimentos de fanerógamas marinas expuestas a vertidos de granjas marinas

Resumen

El enriquecimiento de nutrientes ha degradado numerosas áreas costeras y aparece como una de las principales causas de la desaparición de fanerógamas a nivel mundial. El análisis de los isótopos estables del nitrógeno ($\delta^{15}N$) en fanerógamas puede detectar nitrógeno procedente de actividades humanas tales como la acuicultura marina en jaulas. Sin embargo, la variabilidad de $\delta^{15}N$ asociada a los diferentes compartimentos de las fanerógamas aún no ha sido estudiada. Por esta razón, en este estudio evaluamos la capacidad de diferentes compartimentos de fanerógamas para responder a la entrada de nutrientes. Para ello comparamos las respuestas de los isótopos estable del N en epífitos, hojas jóvenes, rizomas y raíces de las fanerógamas marinas *Posidonia oceanica* (L.) Delile y *Cymodocea nodosa* (Ucria) Ascherson con el fin de distinguir entre las respuestas de las diferentes partes de la planta. Este estudió se desarrolló en un gradiente de disponibilidad de nutrientes derivado de tres granjas marinas localizadas en diferentes sitios de la costa Española. Se observaron diferencias significativas en la composición isotópica en función de la fracción de la planta analizada, siendo los epífitos, el compartimento analizado con una mejor respuesta a la presencia de N procedente de granjas amrinas, seguido de los rizomas. Este studio demuestra la utilidad del $\delta^{15}N$ en epífitos y rizomas como indicadores de la presencia de N procedente de las actividades de acuicultura. Por lo tanto, se sugiere el uso de estos dos tejidos para monitorizar incremento/descensos de nitrogeno derivado de actividades humanas.

Abstract

Nutrient over-enrichment has degraded many coastal waters and has been invoked as a major cause of seagrass disappearance worldwide. The analysis of nitrogen stable isotopes ($\delta^{15}N$) in seagrasses can detect environmental nitrogen derived from human activities, including those generated by fish farms. However the variability of $\delta^{15}N$ associated to different compartments of seagrasses has not been investigated. By this reason, in this study we report on the capacity of different seagrass compartments to respond to nutrient inputs. To accomplish this goal, we compared the responses of nitrogen stable isotopes of seagrass epiphytes, young leaves, rhizomes and roots in the seagrasses *Posidonia oceanica* (L.) Delile and *Cymodocea nodosa* (Ucria) Ascherson in order to distinguish between plant part responses. We performed our study in a gradient of nutrient availability derived from three offshore fish farms located in different sites of the Spanish coast. Significant differences in the isotopic composition were observed as a function of plant fraction being seagrass epiphytes the analyzed tissue with a better response to nitrogen derived from fish farm, followed by the rhizomes. This study demonstrates the usefulness of $\delta^{15}N$ in epiphytes and rhizomes of seagrasses as indicators nitrogen derived from aquaculture activities. Therefore we suggest the use of these two tissues to monitor time-integrated decrease/increase of nitrogen derived from human activities.

Keywords: Nitrogen Stable isotopes ($\delta^{15}N$), Seagrasses, Fish farm, Indicators.

Introduction

In the last decades the coastal populations has grown rapidly increasing considerably the human pressures and impacts on marine coastal ecosystems (Albayrak et al., 2006; UNEP, 2007; Halpern et al., 2008). In particular, the potential of aquaculture wastes to alter environmental conditions and benthic communities is an issue of special concern for marine scientist and coastal managers (Pergent-Martini et al., 2006; Primavera, 2006; Holmer et al., 2008). In fact, there is increasing evidence that off-shore aquaculture can compromise the conservation of sensitive and valuable nearshore habitats such as seagrass communities, due to the dispersion of particulate and dissolved wastes over a wide range of spatial scales (e.g. Delgado et al., 1997; Sarà et al., 2006, Dolenec et al., 2007; Ruiz et al., in press).

Seagrass meadows are considered one of the major marine ecosystems of many tropical and temperate coastal areas worldwide, where they are experiencing a significant decline in the last decades mostly related to human impacts (Short and Wyllie-Echeverria, 1996). Aquaculture wastes has been reported as one of the most frequent cause of seagrass deterioration or lost from estuaries to open marine systems (Ruiz et al., 2001; Pergent-Martini et al., 2006; Pérez et al., 2007; Holmer et al., 2008), due to the disruption of ecosystem structure and function caused by nutrient enrichment (Burkholder et al., 2007). Therefore, since seagrass communities are habitats of great ecological and economical relevance (Hemminga and Duarte, 2000), the detection of nutrient-induced effects in these complex ecosystems is critical for the proper management and control of environmental impact of aquaculture activities.

Increase in N availability is one of the major consequences of human eutrophication in seagrass coastal ecosystems (Short and Burdick, 1996; Touchette and Burkholder, 2000). Both N content and N stable isotope ($\delta^{15}N$) measured in seagrass tissues has been shown to increase in response to increased nutrient loads of anthropogenic origin (e.g. Costanzo et al., 2001; Yamamuro et al., 2003; Lepoint et al., 2004, Udy and Dennison, 1997, Lepoint et al., 2008) and

hence they has been extensively used as bioindicators of the effects of aquaculture wastes on coastal ecosystems (Jones et al., 2001; Vizzini and Mazzola, 2004, 2006; Perez et al., 2008). However, variability of nitrogen in seagrass tissues is not only governed by water column nitrogen, but also by complex internal regulation (translocation, transformation, storage) and sediment pools (Invers et al., 2004; Evrard et al., 2005; Romero et al., 2006). At the individual level, N concentrations and isotopic signatures also depend on the organ type and age (Vizzini et al., 2003; Yamamuro et al., 2004). As consequence, seagrass tissues can be less sensitive to external nutrient concentrations (e.g. Lepoint et al., 2003; Lee et al., 2004) relative to macroalgae or epiphytes which generally depend more on the water column nitrogen. This differential sensitivity of seagrass compartments has been rarely considered in the assessment of farm wastes effects on seagrass communities (Pérez et al., 2008), but it must represent a critical point for the use of these variables as seagrass bioindicators of aquaculture impacts. Accordingly, the aim of the present study was to evaluate the seagrass compartment that better provide clearer and reliable information about the impact of farm wastes on seagrass communities. To this end, we compared the variability of total N content and $\delta^{15}N$ signature in seagrass organs (leaf, rhizome and roots) and leaf epiphytes between healthy, unpolluted meadows and nearby meadows under the influence of fish farm wastes.

Materials and Methods

Study sites and sampling design

Three study sites were selected located in different geographic areas of the Spanish coast: one in the Atlantic Ocean, in the coast of the Canary Island (Tenerife) and two in the Mediterranean Sea, in the coast of Catalonia (Tarragona) and Murcia (Murcia) (Fig. 1). All these sites are characterized by the presence of off-shore fish farm facilities placed in front of large seagrass

meadows of the dominant species of each geographic area (*Posidonia oceanica* in the Mediterranean Sea and *Cymodocea nodosa* in the Atlantic Ocean). Each study case differed in husbandry parameters (fish farm production and cultivated species) and seagrass characteristics (meadow structure variables: shoot density and meadow cover) as indicated in Table 1. At these study sites, previous studies demonstrated the existence of a gradient of dissolved N between fish cages and the seagrass meadow, which make them suitable for the aim of the present study. These studies form part of a project aimed to characterize the spatial extent of fish farm effluents by analyzing N content and $\delta^{15}N$ measured in macroalgae tissues placed in pelagic bioassays deployed at increasing distances from fish cages (Garcia-Sanz et al., in press).

The study was carried out between July and August in 2006, which is the time of the year when both seagrass and its epiphytic community reach its maximum seasonal development (Romero et al., 1989) and when fish farm production (and wastes) are at its maximum. We limited the study to that narrow period in order to avoid seasonal variability of seagrass N content and $\delta^{15}N$ reported for these seagrass species (Vizzini et al 2003). At each geographic location, two meadow areas were selected at similar depths (Table 1): 1) A 'Fish farm meadow area' (FA), which correspond to the meadow area situated just in front of the fish farm, and 2) a 'Control meadow area' (CA), which correspond to a meadow without aquaculture waste influence. In each case, both FA and CA meadows did not showed significant differences in meadow structure characteristics (i.e. shoot density and meadow cover, Table 1), which corresponded to those described for meadows with a good ecological status at the same geographical area and comparable depths (Pers. Obs.). Distances between fish farm sampling area and fish farm facilities were of 2.2 km in Canary Island, 2.8 Km in Murcia and 0.2 km in Catalonia.

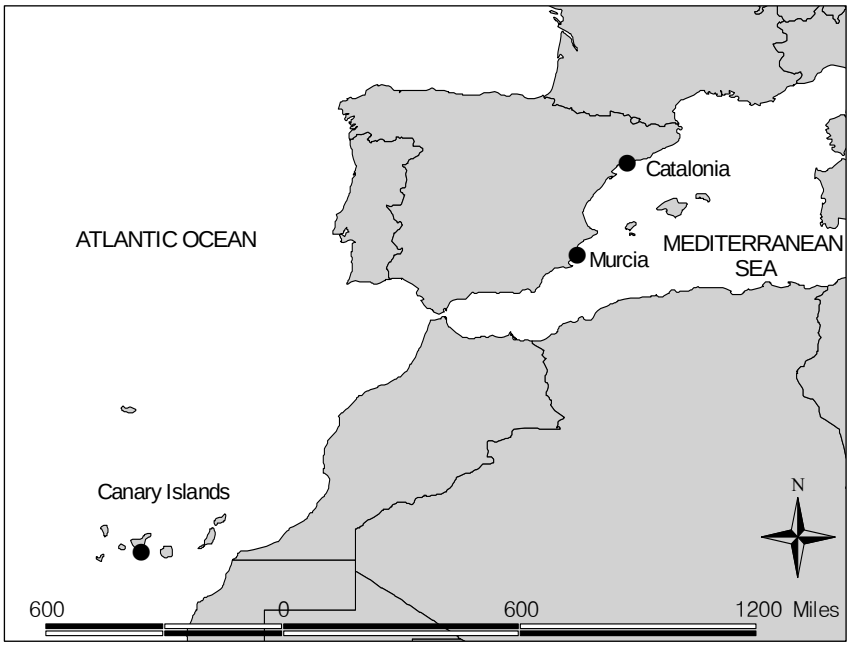


Figure 1: Sample sites map

In order to asses small-scale spatial variability of the measured variables within each meadow area (FA and CA), two sampling stations separated by a distance of 100-200 meters among them were randomly selected. In each sampling station, all shoots contained in six 625 cm^2 quadrates randomly placed within the meadow were collected. In the laboratory, for each sample, epiphytes and seagrass tissues (leaves, rhizomes and roots) were separated, dried at 50 ºC until constant weight (48 h) and ground until obtain a fine powder. For *P. oceanica* only young leaves (0-50 days-old) and the first 4 cm of rhizomes were used to avoid ageing effects. For analysis of total N and stable isotopic ratio dry ground samples were weighted (2.5 µg) and put into tin capsules.

Table 1. Characteristics of the fish farms and the seagrass meadows in the three locations of study.

	Canary Islands		**Murcia**		**Catalonia**	
1. Husbandry parameters:						
• Fish production (tons $year^{-1}$):	375		6,197		800	
• Cultivated species:	sea bream (*Sparus auratus*) sea bass (*Dicentrarchus labrax*)		blue fin tuna (*Thunnus thynnus*) sea bream (*Sparus auratus*) Meagre (*Argyrosomus regius*) sea bass (*Dicentrarchus labrax*)		sea bream (*Sparus auratus*)	
2. Seagrass meadow characteristics:	fish farm area	Control area	fish farm area	control area	fish farm area	Control area
• Seagrass species:	*Cymodocea nodosa*		*Posidonia oceanica*		*Posidonia oceanica*	
• Sampling depth (m):	22.3	22.8	27	26.3	15	18
• Shoot density (nº shoot m^{-2}):	545.5 ± 63.0	605.8 ± 59.7	154.2 ± 8.9	231.3 ± 30.3	-	496.1 ± 78.4
• Meadow cover (%):	42.5 ± 2.2	58.5 ± 6.1	17.6 ± 1.2	21.1 ± 1.9	-	46.6 ± 11.4

Analytical procedures

The N isotopic analysis was performed using an elemental analyzer ThermoFinnigan FlashEA1112 and a mass spectometer DELTAplus with an interface of continuous flow ConFlo II of Finnigan MAT that determine the isotopic composition. The isotopic data was reported in common delta units referred to atmospheric nitrogen standards using the following equation (Peterson and Fry, 1987):

$$\delta^{15}N\ (‰) = (R_{sample} / R_{standard}) - 1) \times 10^3 \qquad \text{where, } R = {}^{15}N / {}^{14}N$$

Statistical analysis

For each study case and plant tissue a one factor nested ANOVA model was used to test the null hypothesis that both total N and $\delta^{15}N$ were similar within and between meadow areas. The model considered 'meadow areas' as a fixed factor with two levels (FA and CA) and 'sampling stations' as a random factor with two levels. Prior to the analysis, homogeneity of variances was tested by Cochran's C-test and data were properly transformed when necessary. Variance components associated to the ANOVA terms were also calculated (Quinn and Keough 2002) for qualitative comparisons of variability patterns between and within meadows. The Statistical software was used to perform the ANOVA analysis.

Results

In the Canary Islands study case, $\delta^{15}N$ values obtained in the *C. nodosa* fish farm meadow area (FA, 3.5-4.0 ‰) were higher that those of the control meadow area (CA, 2.2-2.9 ‰) (Fig. 2), although such differences were significant only in epiphytes and leaves of *C. nodosa* (Table 2). This $\delta^{15}N$ enrichment was more pronounced in epiphytes (27 % higher in FA than in CA) than in leaves (16.6 %). Rhizomes and roots also showed higher mean values in the FA than in CA (35.4 and 24 %, respectively), but it were not statistically significant in this case. In the Murcia study case, mean $\delta^{15}N$ values obtained in *P. oceanica* tissues and epiphytes of CA (3-4.2 ‰) and FA (3.7-4.8 ‰) meadow areas were higher than those measured in the Canary Island study case, although the pattern of variation between meadow areas showed by the different *P. oceanica* compartments was almost identical to that described for the Canary Island study case (Figure 2 and Table 2). In Murcia, the magnitude of the $\delta^{15}N$ enrichment of leaf and epiphytes in the FA meadow was quite similar (13-19%). In the Catalonia study case, $\delta^{15}N$ mean values were the highest obtained in this study (4.6-5.6 ‰; Fig. 2); these values are also higher than

those reported for *P. oceanica* tissues and epiphytes of unpolluted meadows throughout the Mediterranean Sea (2.1-4 ‰; Vizzini and Mazzola 2004; Pérez et al 2008), which are similar to those reported for the CA meadow at the Murcia study case. In Catalonia, only a small (but significant) increase of mean $\delta^{15}N$ value was detected in epiphytes of the FA meadow area (9.4 % increment), relative to those obtained in the CA meadow (Table 2).

Table 2. Summary of nested ANOVA results and estimated variance component (% var.) on nitrogen stable isotope ratios ($\delta^{15}N$) in different seagrass tissues and epiphytes collected in Canary Islands, Murcia and Catalonia. The table shows the differences between Areas (Control and Fish farm Area) and Sites within Areas. H_0: There are not significant different between sites and areas. NS: not significant, **p*<0.05; ***p*<0.01; ****p*<0.001.

	Canary Islands					Murcia					Catalonia				
Epiphytes	df	MS	F	% var	P	df	MS	F	% var	P	df	MS	F	% var	P
Area	1	6.28	994.0	80.3	***	1	2.35	31.2	70.2	*	1	0.84	19.4	62.5	*
Site (Area)	2	0.01	0.099	0.0	ns	2	0.08	2.00	0.0	ns	2	0.04	0.99	0.0	ns
residual	18	0.06		19.6		20	0.04		29.7		8	0.04		37.5	
Leaf	df	MS	F	% var	P	df	MS	F	% var	P	df	MS	F	% var	P
Area	1	2.61	131.8	29.6	**	1	3.03	48.4	67.3	*	1	0.004	0.13	0.0	ns
Site (Area)	2	0.02	0.077	0.0	ns	2	0.06	1.02	0.0	ns	2	0.032	0.78	0.0	ns
residual	20	0.26		70.3		20	0.06		32.6		8	0.042		100.0	
Rhizome	df	MS	F	% var	P	df	MS	F	% var	P	df	MS	F	% var	P
Area	1	9.29	5.66	45.5	ns	1	8.67	4.52	39.5	ns	1	0.34	1.71	8.5	ns
Site (Area)	2	1.64	7.02	18.4	**	2	1.92	4.61	10.6	*	2	0.2	1.77	10.9	ns
residual	19	0.23		36.0		20	0.41		49.7		8	0.11		80.4	
Root	df	MS	F	% var	P	df	MS	F	% var	P					
Area	1	4.26	5.230	29.4	ns	1	3.13	6.87	9.3	ns					
Site (Area)	2	0.81	3.048	10.0	ns	2	0.45	0.43	0.0	ns					
residual	19	0.27		60.5		20	1.06		90.7						

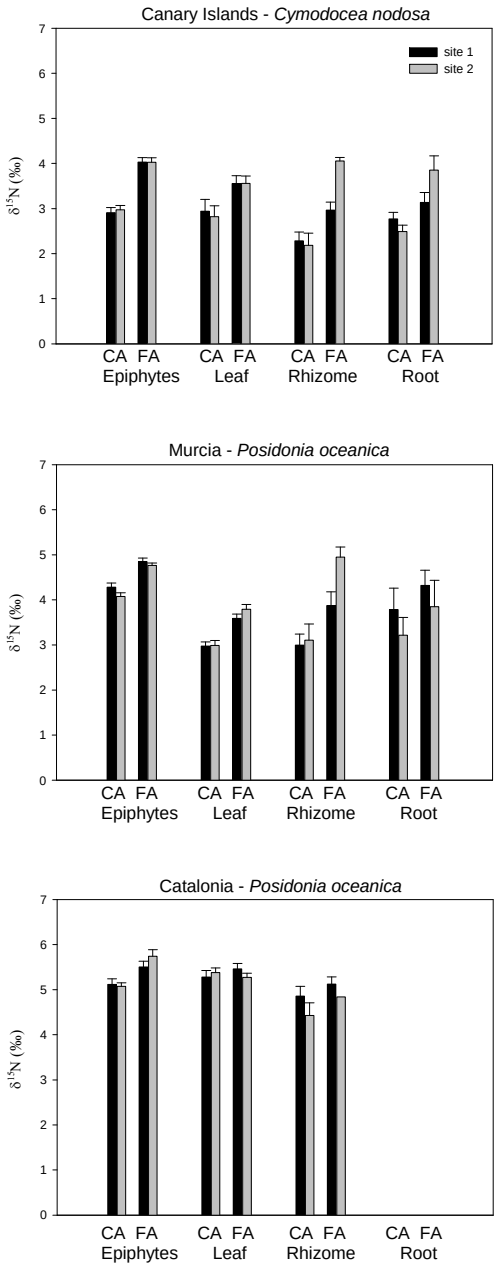


Figure 2: Mean nitrogen stable isotope ratios ($\delta^{15}N$) values in different seagrass tissues and epiphytes collected in Canary Islands, Murcia and Catalonia in Control and Fish farm Areas (CA and FA). Vertical bars correspond to the standard error of the mean.

Mean values of total N content ranged between 1.1 and 1.8 % in the CA meadow area of Canary Island, with higher values observed for *C. nodosa* leaf and root tissues (Fig. 3). Values of this variable significantly increased in epiphytes and rhizomes of the FA meadow area relative to the CA area (42.6 and 41.7 %, respectively; Table 3). Differences between meadow areas were also significant for roots (Table 3), but it showed an opposite pattern to that described for epiphytes and rhizomes (Fig. 3). Both in Murcia and Catalonia study cases, total N content of the *P. oceanica* compartments analyzed ranged between 0.5 and 2 %, although higher mean values were recorded in rhizomes of the FA meadow area in Murcia (Fig. 3). In both cases, higher mean values were observed for leaf and rhizome tissues while in epiphytes were always below 1 %. Also in both cases, no significant differences were found for this variable between meadow areas for any seagrass compartment (Table 3).

Variance components calculated for each seagrass compartment and study case are showed in Tables 2 and 3. Both for N and $\delta^{15}N$, the proportion of variance accounted by the 'meadow area' effect represented one of the most important contribution to the total variance (25-80%) in those cases where differences between meadow areas were found significant. Variance accounted by the residual term (i.e. samples within sites) was also relevant, but in all analyzed cases (19-100%). Variability between sites within meadow areas (i.e. the nested factor) was very low or negligible in most cases (0-10 %), but it was more important (up to 60%) and/or significant for a limited number of cases ($\delta^{15}N$ of rhizomes in Canary Island and Murcia, N content of leaf and rhizomes of Murcia and leaf N content in Catalonia). Since the variance of the nested factor 'Site' is the denominator in F-ratio of the 'Area' effect in this ANOVA model, this local source of heterogeneity can prevent the detection of significant differences between meadow areas even when these differences are apparent, as it was the case for N isotopes in rhizomes from Canary Island and Murcia (Fig. 2, Table 2).

Table 3. Summary of nested ANOVA results and estimated variance component (% var.) on total nitrogen content (% N) in different seagrass tissues and epiphytes collected in Canary Islands, Murcia and Catalonia. The table shows the differences between Areas (Control and Fish farm Area) and Sites within Areas. H_0: There are not significant different between sites and areas. NS: not significant, *p<0.05; **p<0.01; ***p<0.001.

	Canary Islands					Murcia					Catalonia				
Epiphytes	df	MS	F	% var	P	df	MS	F	% var	P	df	MS	F	% var	P
Area	1	4.87	77.4	68.6	***	1	0.0004	0.04	0.0	ns	1	0.09	2.81	20.0	ns
Site (Area)	2	0.06	0.67	0.0	ns	2	0.010	0.58	0.0	ns	2	0.03	1.85	0.0	ns
residual	18	0.09		31.3		20	0.017		100.0		8	0.02		80.0	
Leaf	df	MS	F	% var	P	df	MS	F	% var	P	df	MS	F	% var	P
Area	1	0.01	0.10	0.0	ns	1	0.026	0.32	0.0	ns	1	0.007	0.36	0.0	ns
Site (Area)	2	0.13	1.58	4.9	ns	2	0.078	11.1	46.4	***	2	0.020	6.74	60.0	*
residual	20	0.08		95.0		20	0.007		53.5		8	0.002		40.0	
Rhizome	df	MS	F	% var	P	df	MS	F	% var	P	df	MS	F	% var	P
Area	1	3.99	57.1	59.7	*	1	0.624	0.58	0.0	ns	1	0.042	0.33	0.0	ns
Site (Area)	2	0.06	0.62	0.0	ns	2	1.058	7.25	30.3	**	2	0.125	1.27	4.7	ns
residual	19	0.11		40.2		20	0.145		69.6		8	0.098		95.2	
Root	df	MS	F	% var	P	df	MS	F	% var	P					
Area	1	0.45	10.6	25.2	*(1)	1	0.013	2.45	0.68	ns					
Site (Area)	2	0.04	0.77	0.0	ns	2	0.005	0.18	0.0	ns					
residual	19	0.05		74.7		20	0.029		99.3						

*(1) significativo tras el pooling del nested factor

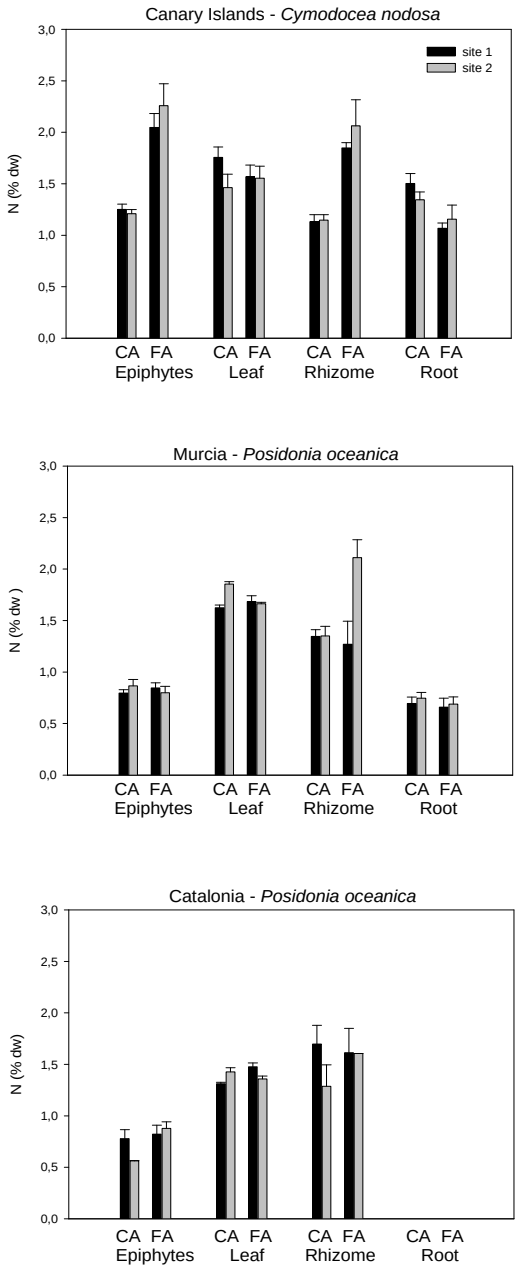


Figure 2: Mean total nitrogen content (% N) in different seagrass tissues and epiphytes collected in Canary Islands, Murcia and Catalonia in Control and Fish farm Areas (CA and FA). Vertical bars correspond to the standard error of the mean.

Discussion

The analysis of total N content and nitrogen stable isotope ratios ($\delta^{15}N$) in seagrass tissues and epiphytes performed in this study revealed differential sensitivity of seagrass compartments to the increase of external nutrient concentrations caused by the dispersion of farm wastes from off-shore aquaculture facilities. $\delta^{15}N$ values showed a consistent and significant increase near fish farms in epiphytes and leaves of *Cymodocea nodosa* in Canary Islands and *Posidonia oceanica* in Murcia, and only in epiphytes in Catalonia. The $\delta^{15}N$ values obtained in these tissues (3.5-5.5 ‰) were similar to the $\delta^{15}N$ values measured in the organic matter derived from fish farms (Sarà et al., 2004) so, we can corroborate that fish farm wastes are the responsible of the $\delta^{15}N$ enrichment in such tissues.

Epiphytes are expected to be more sensitive to changes in water column nitrogen than seagrass tissues since they are able to exploit dissolved nitrogen sources more rapidly than their host plant (Lepoint et al., 2007). Furthermore, epiphytic algae have a higher nutrient demand (i.e., lower C/N/P ratio) (Pedersen and Borum 1997) since they only have access to water-column nutrient. Our results support this contention since epiphytes were the only seagrass compartment analyzed that showed significant differences in $\delta^{15}N$ values between fish farm and control areas in all the study cases (Canary Islands, Murcia and Catalonia).

Leaves showed significant differences in $\delta^{15}N$ values between fish farm and control areas in all cases, except in Catalonia. Significant internal variations in $\delta^{15}N$ of seagrass leaves related to the complex internal regulation of leaf nitrogen in seagrasses (Invers et al., 2004) could explain the lack of response in Catalonia as well as that found in others studies (Yamamuro et al., 2004). It make difficult to do any simple assumption about plant nutrient status by means of only this tissue. However, actually, leaves is the tissue more widely used in ecological studies to evaluate nutrient enrichment using the isotopic content of seagrasses (Grice

et al., 1996; Fourqurean et al., 1997; Costanzo et al., 2001; Jones et al., 2001; Yamamuro et al., 2003; Vizzini and Mazzola, 2004, 2006).

In rhizomes and roots we found no significance differences in $\delta^{15}N$ values between the area influenced by fish farm wastes and the control area. However, the mean values were clearly higher near the fish farms than in the control area in several study cases, especially in rhizomes. These cases were the rhizomes and roots in the Canary Island and the rhizomes in Murcia. In all of them, the variability associate to the nested factor (i.e. spatial variability inside of each meadow) was high (10-19 %). This variability was associated mainly with the meadows located near fish farms probably by the different influence of the waste in the meadow due to local hydrodynamic factor. So, this high variability of the nested factor could have masked the increases in $\delta^{15}N$ values near fish farm. Probably, an experimental design with more replicates of sites inside each meadow could reveal significance differences, specially in rhizomes, since the high storage capacity of this organ (e.g. Invers et al. 2004) makes the rhizomatic N pool sensitive to changes in N availability (Martínez-Crego et al., 2008). In fact, increases in $\delta^{15}N$ in this organ have been selected as descriptor in a multivariate index (POMI) to asses the ecological status of coastal waters based on the seagrass *P. oceanica* (Romero et al., 2007). Roots response could be more variable, as occurs in leaves, because of the complex and variable strategies of seagrasses for meeting nitrogen requirements involving both leaf and root uptake and internal resorption (Touchette and Burkholder, 2000).

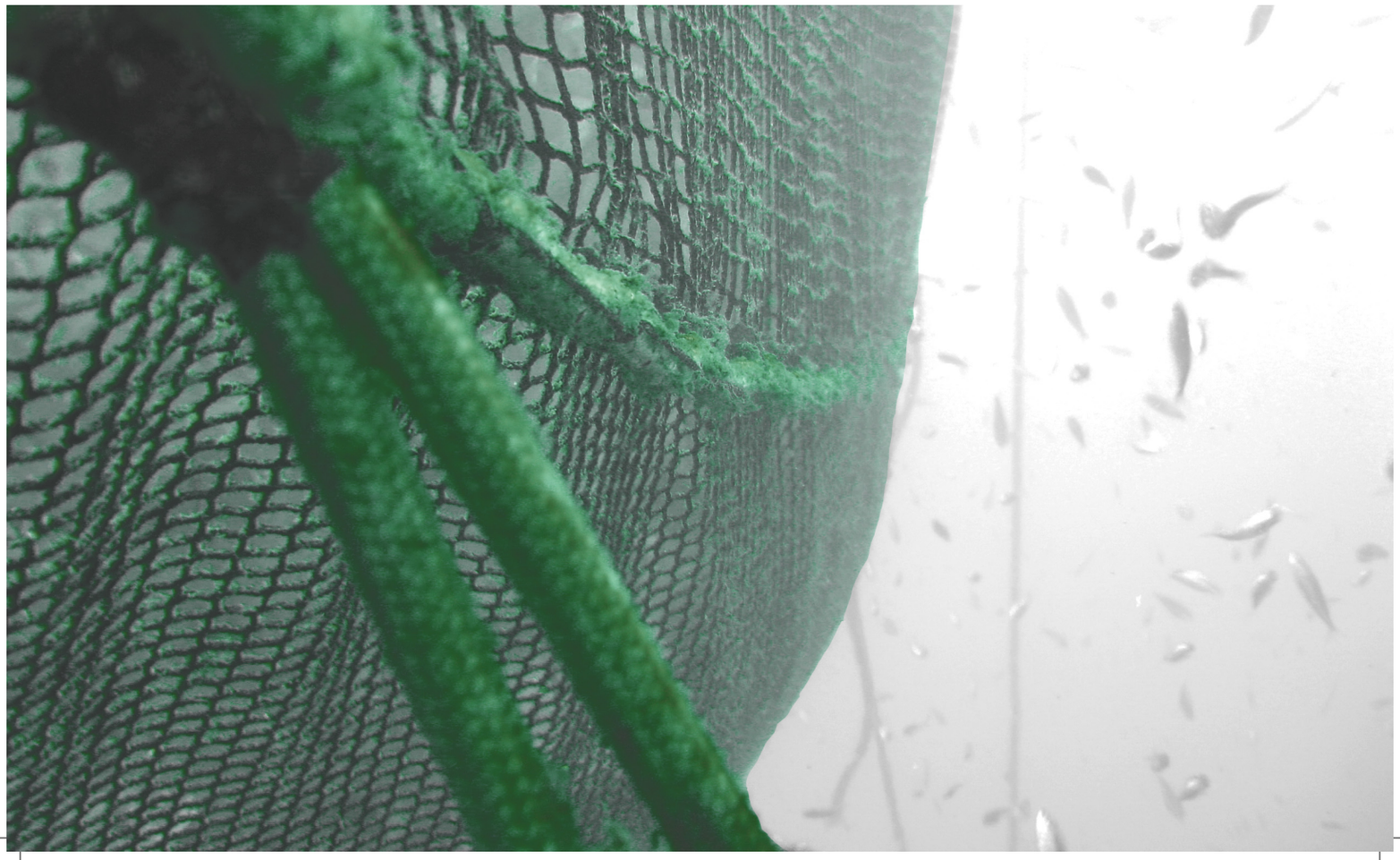
On the other hand, when we compare the results obtained from the analysis of $\delta^{15}N$ in seagrass tissues with the total nitrogen content (% N) in plant tissues we clearly appreciate that the isotopic analysis is better than the analysis of nitrogen content to detect the dissolved nitrogen derived from fish farms. Total nitrogen content increased significantly near fish farms only in two cases, epiphytes and rhizomes in Canary Islands. This indicates that, probably only in the Canary Islands, the most oligotrophic study site analyzed, the N derived from fish farm arrives in excess to the meadow. In Murcia and Catalonia, the N derived from fish farm also

arrives to the meadow, due to the positive response of $\delta^{15}N$ obtained in epiphytes. However, there are a lack of response of % N in all the tissues analyzed. It could be explained by several reasons. By one hand, the dilution and dispersion of the nitrogen due to local regimen of currents can avoid nitrogen accumulation in seagrass tissues (Sarà et al., 2006). Then, the N supplied to the meadow could be as low as to merit long-term N storage and are likely used to sustain growth (McGlathery 1995; Udy and Dennison, 1997; Lin and Fong 2008). On the other hand, kinetics parameters are highly variable among seagrass species (Romero et al., 2006). Therefore differences in nutrient economy between *C. nodosa* and *P. oceanica* could have had influence in the results (Invers et al., 2004). In addition, nutrient enrichment could favour opportunistic epiphytic algae over seagrass leaves (Duarte 1995, McGlathery et al., 1995, Frankovich and Fourqurean, 1997; Wear et al., 1999) which rapid growth prevents for N accumulation in seagrass tissues. Finally, increases in % N in seagrass tissues could not be detected due to the natural variability of this variable between individual plants (residual) or parts of the meadow (nested factor).

In spite of carry out experiments in different regions and with two seagrass species, our results verify the role of the analysis of the $\delta^{15}N$ in epiphytes, and seagrass tissues in comparison with the use of total N content to detect rapidly nitrogen derived from aquaculture activities in seagrasses avoiding changes in the community with drastic effects for the survival of the meadow. So we propose the analysis of nitrogen stable isotope ratio, especially in seagrass epiphytes and rhizomes, to monitoring dissolved nitrogen derived from human activities such as offshore aquaculture.

Chapter 3

An evaluation of a macroalgal bioassay tool for assessing the spatial extent of nutrient release from offshore fish farms



Evaluación de una herramienta basada en bioensayos con macroalgas para estimar la extensión espacial de nutrientes liberados por jaulas marinas de cultivo

Resumen

En este studio se ha desarrollado y evaluado una herramienta basada en bioensayos con macroalgas para monitorizar el alcance especial de los vertidos disueltos liberados de granjas marinas *off-shore* en ecosistemas costeros marinos. Esta herramienta analiza el ratio isotópico del nitrógeno ($\delta^{15}N$) en tejidos de varios productores primarios bentónicos (macroalgas y epífitos de *P. oceanica*) incubados en la columna de agua a distancias crecientes (de 0 m a 1000 m) de las granjas marinas a través de dispositivos de incubación. Los bioensayos se aplicaron en tres granjas marinas situadas en diferentes localidades geográficas (Islas Canarias, Murcia y Cataluña) y se probó: la capacidad de respuesta de las diferentes especies de macroalgas usadas en relación a su resistencia y sensibilidad a vertidos de granjas marinas, la profundidad de incubación (superficie o fondo) y tiempo de incubación (2, 4 o 6 días) más adecuados para detectar la distribución espacial de $\delta^{15}N$ de las inmediaciones de las granjas marinas. En términos generales, los resultados mostraron un incremento gradual y significativo de los valores de $\delta^{15}N$ hacia las jaulas de cultivo con respecto a los valores de referencia (iniciales) y los valores control en todas las especies de macroalgas evaluadas, excepto en el alga roja *Asparagopsis taxiformis* de las Islas Canarias. Los resultados obtenidos variaron en función de las condiciones experimentales analizadas y del régimen de nutrientes de cada area costera. El gradiente espacial fue más consistente en la parte somera de la columna de agua que en la profunda y fue estadísticamente significativo tras un periodo de incubación de cuatro días. Estos resultados confirman la efectividad y fiabilidad del método propuesto pudiéndose por tanto aplicar para evaluar la extensión espacial de los nutrientes derivados por las granjas marinas en

los programas de monitorización de granjas marinas.

Abstract

In this study we develop and evaluate a macroalgal bioassay tool for monitoring the spatial extent of dissolved wastes loaded from offshore fish farms into the marine coastal ecosystem. This tool is based in the analysis of the nitrogen stable isotope ratio ($\delta^{15}N$) in tissues of several benthic primary producers (macroalgae and *P. oceanica* epiphytes) incubated in the water column at increasing distances (from 0 m to 1000 m) from the fish cages by means of incubation devices. The bioassays were performed in three fish farms situated in different geographical locations (the Canary Islands, Murcia and Catalonia) and we test: the suitability of the different macroalgae species used in relation with their resistance to incubation and their sensitivity to fish-farm wastes and the most adequate incubation depth (shallow or depth) and incubation time (2, 4 or 6 days) to detect the spatial distribution of $\delta^{15}N$ around fish farms. In general terms, the results showed a gradual and significant increment of $\delta^{15}N$ values towards the fish cages with respect to the reference (initial) and control values for all the species of macroalgae tested except for the red algae *Asparagopsis taxiformis* from Canary Islands. The magnitude and shape of the reported spatial responses varied in function of the experimental settings analyzed as well as in function of the nutrient regime characteristics of each coastal area. The spatial gradient was more consistent in the shallow part of the water column, than in the deeper part and was statistically significant after an incubation period of four days. These results confirm the effectiveness and reliability of the method proposed, that could be applied to assess the spatial extent of nutrients derived from fish farm to use in monitoring programs in offshore fish farms.

Keywords: Nitrogen Stable isotopes ($\delta^{15}N$), Fish farms, Macroalgae, Bioassays, Indicators.

Introduction

The use of floating net cages in marine aquaculture has grown steadily over recent years in the Mediterranean (FAO, 2007). This development has sparked much concern over the threats posed by fish-farm wastes entering marine ecosystems. Fish-farm waste mainly composed of uneaten food and fish waste (excreta and faeces), increases the concentration of particulate organic mater (POM) and dissolved nutrients in the surrounding waters (Pitta et al., 1999; Karakassis et al., 2001; Norvdarg and Johansson, 2002). Particulate waste settles over the seabed and subsequently enriches the sediments (Vezzuli et al., 2002); resuspension of particulate materials from sediments further contributes to the increase in POM and dissolved nutrients in the water column (Holby and Hall, 1991; Hall et al., 1992; Christensen et al., 2000). These enrichment of sediments and water-column has lead to significant alterations of benthic marine communities, mainly in soft-bottom macro- and meio-benthos (Mazzola et al., 2000; Mirto et al., 2002) but also in more vulnerable and valuable habitats, particularly seagrasses (Pergent et al., 1999; Ruiz et al., 2001; Holmer et al., 2003; Pergent-Martini et al., 2006; Holmer et al., 2008; Ruiz et al, in press). Most of these negative effects have been reported in shallow and sheltered nearshore environments and, as consequence, most of fish farm facilities are presently placed in deeper, offshore waters, where is presumed to have a lower resident time of aquaculture wastes minimizing impact on benthic communities (Wu et al., 1994; McGhie et al., 2000; Alongi et al., 2003).

Despite the reported evidences of environmental degradation caused by aquaculture activities, there are currently no specific and reliable tools to provide ecologically meaningful information about the impact of fish-farm wastes on marine ecosystems. Classic descriptors based on water-column and sediment physicochemical variables (i.e. DIN, POM, among others) have been systematically used in monitoring programs of aquaculture impacts to assess the spread of fish-farm wastes in marine environments (Dosdat et al., 1995; Roque d´Orbcastel et al., 2008). However, these methods have usually failed to detect nutrient enrichment on

receiving waters surrounding fish farms underestimating the spatial extent of the dissolved fraction of aquaculture wastes (Jones et al., 2001 and references therein). This lack of response is attributed to the fast dispersal of dissolved nutrients, its assimilation by organisms or lost to the atmosphere through volatilization and denitrification (Pitta et al., 1999; Wolanski et al., 2000; Karakassis et al., 2001; Sarà, 2007). This is especially true in offshore environments where the dilution and spread of released nutrients is faster than in more confined sites. Biological indicators (bioindicators) represent an alternative to physical and chemical analyses to assess water quality since they can integrate both persistent and pulsed changes in external nutrient availability over time, reflecting the nutrient regime of the environment and the assimilation of nutrients within the system (Lyngby 1990; Costanzo et al., 2000; Jones et al., 2001). As a result there is now a call for the development of monitoring tools based on bioindicators to properly manage this activity.

Benthic primary producers (macroalgae, seagrasses and epiphytes) are considered excellent potential bioindicators of nutrient availability for several reasons: they are widely distributed and abundant, their biomasses persist over relatively long periods, and they show well-characterized physiological responses to nutrient exposure (Udy and Dennison, 1997; Lyngby et al., 1999). Indeed, the number of studies that use these organisms to assess the influence of external sources of nutrients on coastal ecosystems has recently increased (Fong et al., 1998; Savage and Elmgren, 2004; Lee et al., 2004; Dalsgaard and Krause-Jensen, 2006; Pérez et al., 2008). On the other hand, in the last decade, the analysis of the isotopic nitrogen ratios ($\delta^{15}N$) in benthic primary producers has been revealed as a technique that is particularly effective in tracing external nutrient inputs from certain human activities (see Lepoint et al., 2004 for a review, Sarà et al., 2004, 2006). Organic loads from urban wastes and aquaculture are enriched in the heavy nitrogen isotope (N^{15}) relative to natural sources (Jones et al., 2001; Costanzo et al., 2003, 2004; Cole et al., 2004, 2005). This enrichment in N^{15} can be detected in macrophyte tissues exposed to wastes since they show only small or no fractionation during nitrogen uptake and assimilation (Gartner et al., 2002; Cohen and Fong, 2005; Deutsch and

Voss, 2006). Moreover, the specificity of the nitrogen isotopic signal in macrophyte species to their sources allow to identify nutrient enrichment caused by different human activities e.g. urban wastes and fish farm effluents.

The analysis of the nitrogen isotopic signal in benthic vegetation has been applied successfully to determine the spatial scale of the dispersal of aquaculture effluents derived from land-based farms (Jones et al., 2001; Vizzini and Mazzola, 2004). However, the distribution of benthic macrophytic communities along the coast is not continuous and it is depth limited so these communities are usually absent from deeper areas where offshore aquaculture is expanding. This fact restricts the use of this kind of biological indicators to quantify the impact of fish farm effluents in open waters. To overcome this problem some researches have analyzed variations of $\delta^{15}N$ in samples of macroalgae collected from natural stands and placed into chambers that are then deployed in any desired location (i.e. bioassays) to detect and map the spatial extent of the influence of sewage outfalls over a large marine coastal area (Costanzo et al., 2001; Deutsch and Fong, 2006). Lin and Fong (2008) faced the same problem to detect the spatial extent of shrimp-farm (land-based) effluents deploying macroalgae in mesh bags staked to sediment in unvegetated bottoms. However, to our knowledge its potential has not still been evaluated in marine offshore fish farms. So, we present here a study that uses pelagic macroalgal bioassays in combination with isotopic analysis to determine the spatial extent of offshore fish-farm wastes in open waters.

This study represents an assessment of a method based on the analysis of $\delta^{15}N$ signatures in tissues of benthic primary producers (macroalgae and *P. oceanica* epiphytes) used as active bioindicators (i.e. bioassays) to determine the spatial extent of dissolved wastes derived from offshore aquaculture facilities. Three case studies (fish farms) were selected in three geographically separated sites of the Spanish coast to perform this study. In each case, bioassays were deployed by means of incubation devices in the water column at distances increasingly further from the fish farm facilities and the effects of several key experimental

settings (species of macroalgae, depth of incubation and duration of incubation) on the variations of $\delta^{15}N$ signatures were analyzed. In addition, spatial variations of $\delta^{15}N$ were compared with those indicated by other measures as total nitrogen and total phosphorus content in plant tissues.

Materials and Methods

Study sites

Three offshore fish farms were selected to perform the study. One was located in waters of the Atlantic coast (Tenerife, Canary Islands), and two in the Mediterranean: one in San Pedro del Pinatar (Murcia Region) and the other in L'Ametlla de Mar (Catalonia Region), on the south-eastern and north-eastern coast of the Spanish Peninsula, respectively (Fig. 1). In the Canary Islands, the annual production of the fish farm during the study period was about 375 tons. The species farmed here were sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). In Murcia, the annual production of the fish farm was about 350 tons. Atlantic blue fin tuna (*Thunnus thynnus*) was farmed in Murcia. However, this facility was at the southern end of a larger complex of seven fish farms. These farms were concentrated in an area of 1.47 km^2 and farmed blue fin tuna, sea bream and sea bass with an annual production of 6,760 tons. On an annual basis, the fish farm in Catalonia produced about 800 tons of sea bream. All fish farms selected were located in soft, unvegetated bottoms between 20 and 40 m deep at more than 1 km from shore. The study was carried out during July and August in 2005.

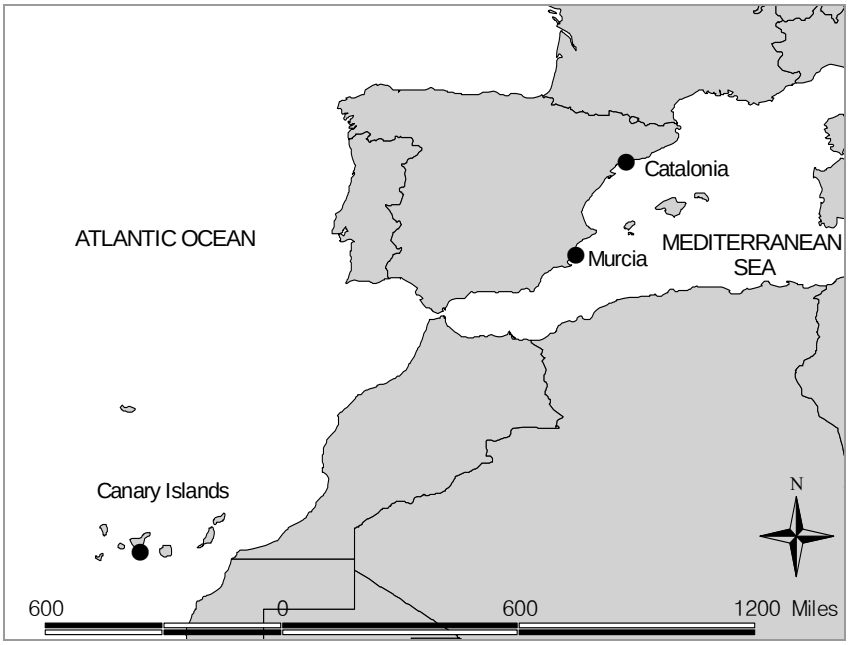


Figure 1: Sample sites map.

Experimental design

At each study site, macroalgal samples were collected from unpolluted sites one day before the bioassay deployment. Macroalgae collected varied in availability in each study site. As a result of this variation, the red algae *Asparagopsis taxiformis* (Delile) Trevisande Saint-Léon and the brown algae *Stypopodium zonale* (Lamouroux) Papenfuss were used in the Canary Islands study site; the brown algae *Dictyopteris polypodioides* (De Candolle) J.V. Lamouroux and the epiphytic community of the seagrass *Posidonia oceanica* (L.) Delile were used in the Murcia fish farm, and the brown algae *Cystoseira mediterranea* (Sauvageau) in the study case of Catalonia. After collection, all macroalgae were maintained until the next day in coolers with well-aerated seawater under low light conditions to avoid further physiological stress by light

and temperature. Ten sub-samples were separated from the bulk of collected plant material and kept frozen until the time of analysis to obtain reference values of the variables analyzed.

The day after sampling, macroalgae pieces (between 25 and 50 g of plant biomass) were placed into incubation devices (comprising plastic 1 cm mesh cylinders measuring 9 cm in diameter and 15 cm in height) and deployed in the water column at sites increasingly further from the fish cages along a 1 km transect in the main current direction (Fig. 2). The direction of the currents during the course of our study was established at each site from data provided by environmental studies available in the respective monitoring programs.

The incubation devices were hung along vertical rope lines deployed at 0, 25, 50, 100, 200, 500 and 1000 meters from the fish cages. Rope lines were attached to a concrete cube weighing 30 kg on the seabed and were maintained in an erect position with a buoy. Along each rope line, three groups of four cylindrical devices (n = 4) were hung at 5 m and at 20 m depth respectively to assess the effect of the incubation depth on the spatial variation of the nitrogen isotopic signal measured in plant tissues. Based in others studies (Beveridge et al., 1994; Wu et al., 1994; Mantzavrakos et al., 2007) that reported that no significant changes in total suspended solids and water transparency were found near offshore fish farms at any of the sites tested, we assume that light intensity is the same in all incubation points at the same depth along the transect .

To evaluate the most adequate incubation time, a group of 4 devices at each depth and each distance (from a total of three groups at each sampling point) was collected at 2, 4 and 6 days after the initial deployment (T2, T4 and T6, respectively). The plant material contained in each device was cleaned with deionized water, stored in plastic bags and kept frozen until analysis.

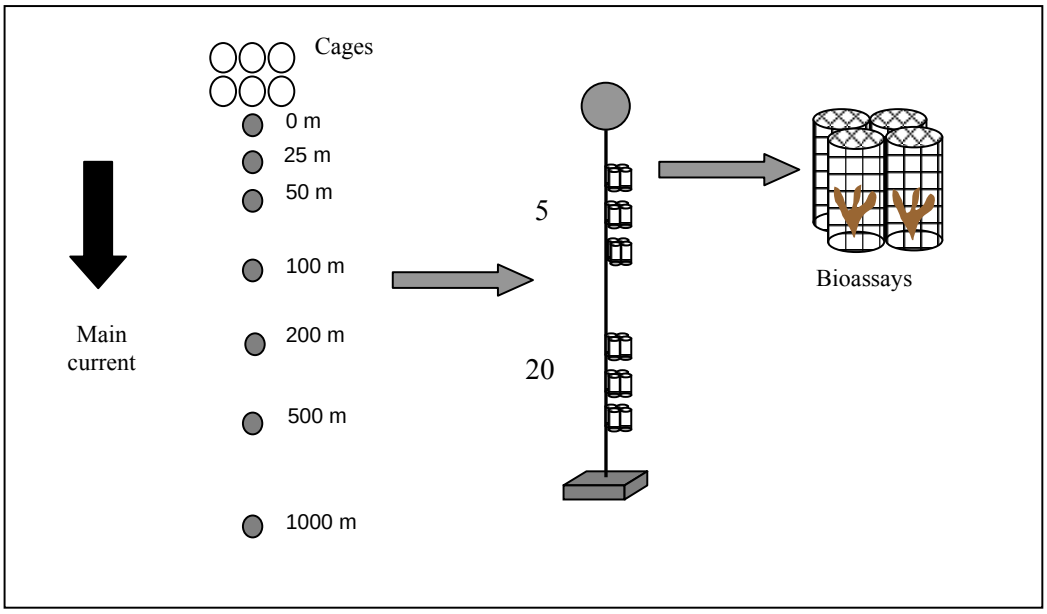


Figure 2: Diagram of the experimental design developed in this study.

Analysis of plant material

Macroalgae samples were dried at 60ºC to constant weight for 24 hours, ground using an agate mortar and pestle and preserved in a desiccator at room temperature. From each sample, between 1.8 and 2 mg of dry weight was encapsulated to determine total nitrogen content and the stable isotope ratio ($\delta^{15}N$).

The isotopic analysis technique was based on the two naturally occurring atomic forms of nitrogen ^{14}N and ^{15}N (Mariotti, 1983). By measuring the ratio of ^{14}N and ^{15}N in dried plant tissue, the relative amount of ^{15}N or $\delta^{15}N$ in the plant can be determined as the relative

difference between the sample and a worldwide standard (atmospheric N_2) using the following equation (Peterson and Fry, 1987):

$$\delta^{15}N = (^{15}N/^{14}N_{sample} / {}^{15}N/^{14}N_{standard} - 1) \times 1000$$

The nitrogen stable isotope ratio ($\delta^{15}N$) and total N content (%) were determined using a continuous flow isotope ratio mass spectrometer (Thermo Finnigan Delta Plus IRMS). This was carried out after combustion in an elemental analyzer Flash EA from TermoFinnigan coupled via a ConFlo II from Finnigan MAT with the elemental analyzer. The ratio $\delta^{15}N$ value was expressed in per mil (‰). The internal laboratory standard employed was acetanilide.

In the Catalonia study, total P (% DW) was also determined in plant material to compare the results obtained with the information provided by the nitrogen variables. Total P content was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) with a vacuum spectrometer (Franson, 1985) following nitric acid-hydrogen peroxide digestion.

Statistical procedures

The effect of distance from fish cages on the dependent variables ($\delta^{15}N$, % N, % P in plant tissues) was analyzed using a separate one-way ANOVA for each combination of depth and incubation time. Before analysis, Cochran's test was used to assess the homogeneity of variances. Differences were considered significant if $p<0.05$. When significant differences were detected a *post* hoc pair-wise comparison of means Student-Newman-Keuls (SNK) test was performed (Zar, 1999). When variances were homogeneous, results were interpreted at the probability level of 0.01 to reduce the possibility of a Type I error (Underwood, 1997). Statgraphics Plus 5.1 data analysis software package was used for the statistical analysis.

Results

Nitrogen Stable Isotope Ratio ($\delta^{15}N$)

Mean $\delta^{15}N$ values obtained at the incubation time T2 for each transects distance were variable and close to the reference values (Figs. 3, 4 and 5 and Table 1). Such variability was significant in most cases but was not influenced by the presence of fish cages (Table 2). As the incubation time increased, differences between bioassays and the reference values increased, the maximum being in the fish cage (0 m) and minimum at the control site (i.e. 1000 m). However, the significance and strength of this spatial trend depended on the incubation time, the macroalgae species used and the depth of the bioassay just as is described below.

Table 1. Measurements of $\delta^{15}N$, % N and % P in macroalgae collected from unpolluted sites at each location

Site	Specie	Reference values		
		$\delta^{15}N$ (‰)	% N	% P
Canary Islands	*Asparagopsis taxiformis*	3.9	1.7	-
	Stypopodium zonale	2.5	0.9	-
Murcia	*Dictyopteris polypodioides*	1.7	1.8	-
	P. oceanica epiphytes	3.5	1.1	-
Catalonia	*Cystoseira mediterranea*	6.1	1.4	0.082

With regard to the time of incubation, significant spatial trends were detected only in the macroalgae incubated for four and six days (T4 and T6, respectively) (Table 2). In the Canary Islands a spatial gradient was only detected by *S. zonale* at T4 (Fig. 3). In the case of *A. taxiformis* variations of $\delta^{15}N$ along the bioassays transect in all times of incubations were not related to the spatial pattern described above, probably due to the deteriorated state of the tissues

of this alga at the end of the experimental period (pigment loss, pers. Obs.) (Fig. 3). In Catalonia the spatial gradient defined by *C. mediterranea* was evident at T4, while it appeared later (T6) in Murcia for the two species analyzed (Figs. 4 and 5).

On the other hand, macroalgae incubated at a depth of 5 m showed a similar response to those incubated at a depth of 20 m (Table 2) although the spatial gradient between the fish farm and control sites was more clearly defined in the upper layer of the water column than at 20 m depth (e.g. *S. zonale, P. oceanica* epiphytes and *C. mediterranea,* Figs. 3-5). All the macroalgae species tested, except for *A. taxiformis*, showed significant spatial trends at a depth of 5 m. At 20 m depth, significant spatial gradients were also found in all macroalgae species, except for *S. zonale* and *P. oceanica* epiphytes (Table 2). Differences between the fish cage (0 m) and control (1000 m) sites were highest for *D. polypodioides* (1.9-fold) and lower for the other species (1.1 to 1.5-fold) (Figs. 3-5); furthermore mean $\delta^{15}N$ values in *D. polypodioides* were highest from initial values (i.e. references values) even at the control site with maximum differences in T6 at a depth of 5 m (Fig. 4).

Table 2 Summary of ANOVA one-way results for $\delta^{15}N$ values of the macroalgae analyzed. *Post hoc* associations are shown in lower-case letters. For the sake of simplicity, only SNK test results for significant differences are shown. H_o: there are no significant effects between distances. NS = not significant, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

Site	Specie	Samples		ANOVA one-way (p-value)	SNK – test (distance)						
		Depth	Time		0	25	50	100	200	500	1000
Canary Islands	*Asparagopsis taxiformis*	5	T2	ns	-	-	-	-	-	-	-
			T4	***	a	a	a	a	b	a	a
			T6	ns (1)	-	-	-	-	-	-	-
		20	T2	**	ab	a	a	a	b	ab	ab
			T4	***	b	ab	a	ab	b	b	c
			T6	ns	-	-	-	-	-	-	-
	Stypopodium zonale	5	T2	***	a	a	b	a	a	a	a
			T4	**	a	a	ab	a	a	ab	b
			T6	*	a	a	a	a	a	a	a
		20	T2	ns	-	-	-	-	-	-	-
			T4	ns	-	-	-	-	-	-	-
			T6	ns	-	-	-	-	-	-	-
Murcia	*Dictyopteris polypodioides*	5	T2	ns (1)	-	-	-	-	-	-	-
			T4	ns	-	-	-	-	-	-	-
			T6	***	a	ab	a	ab	bc	c	d
		20	T2	*** (1)	a	b	b	b	a	b	b
			T4	***	a	b	b	b	b	c	c
			T6	***	a	a	b	b	b	c	d
	Posidonia oceanica epiphytes	5	T2	***	a	b	b	b	b	b	b
			T4	**	a	ab	b	ab	ab	b	b
			T6	***	a	a	a	a	ab	c	bc
		20	T2	***	a	b	b	b	b	b	b
			T4	ns (1)	-	-	-	-	-	-	-
			T6	***	a	bc	bc	ab	bc	c	c
Catalonia	*Cystoseira mediterranea*	5	T2	***	b	a	b	b	a	b	b
			T4	***	a	a	b	b	b		b
			T6	** (1)	a	ab	ab	b	b	b	b
		20	T2	***	b	a	ab	b	b	ab	b
			T4	***	a	a	b	ab	c		bc
			T6	ns	-	-	-	-	-	-	-

(1) Non homogeneous variances. Significant differences if p ≤ 0.01

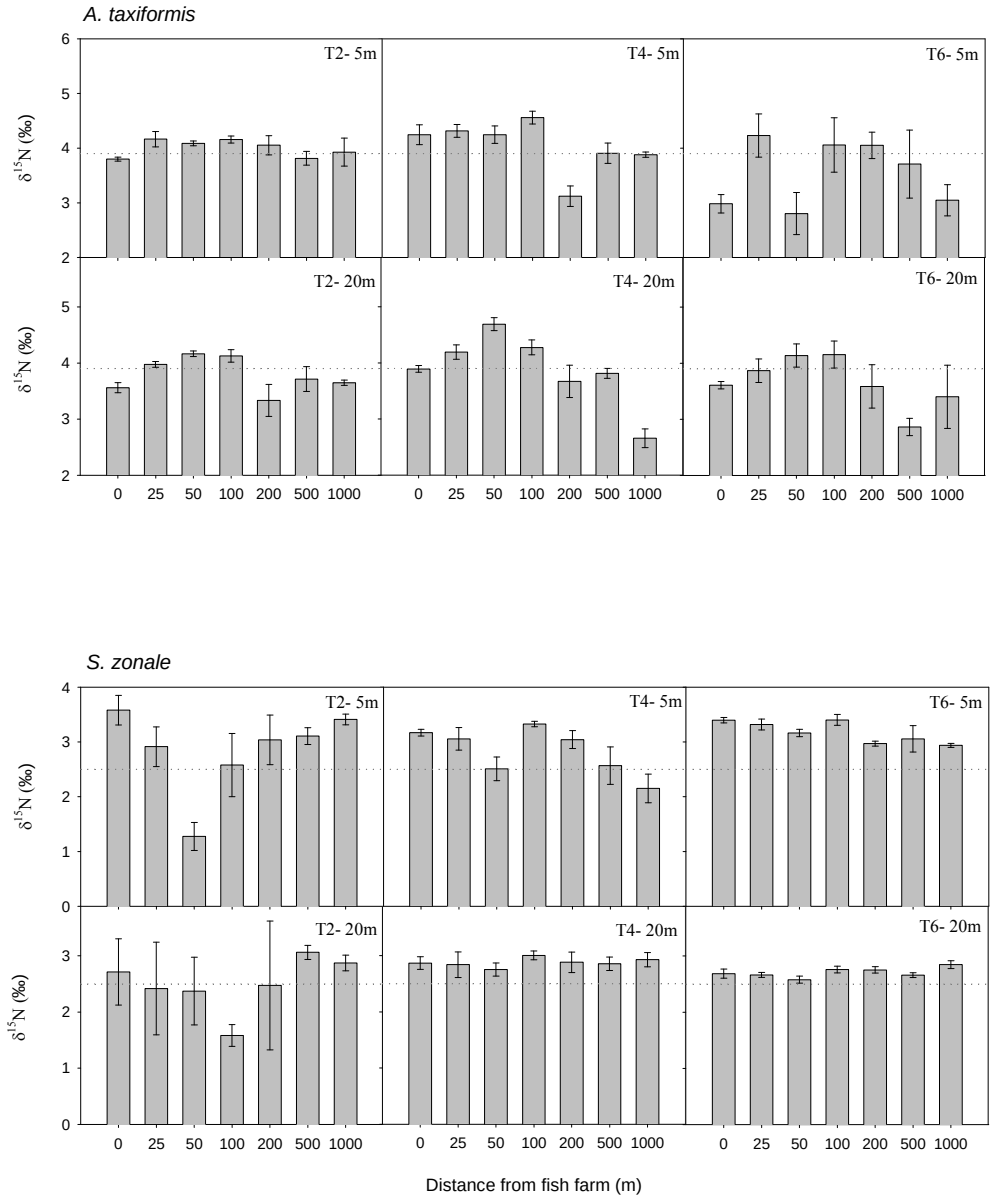


Figure 3: Nitrogen isotopic ratio ($\delta^{15}N$) in the two macroalgae analyzed in Canary Islands, *Asparagopsis taxiformis* and *Stypopodium zonale*. For each species of macroalgae the results for two depths (5-20m) and three times of incubations (T2= 2 days; T4= 4 days; T6= 6 days) at different distances from fish farm are shown. Vertical bars correspond to the standards error of the mean.

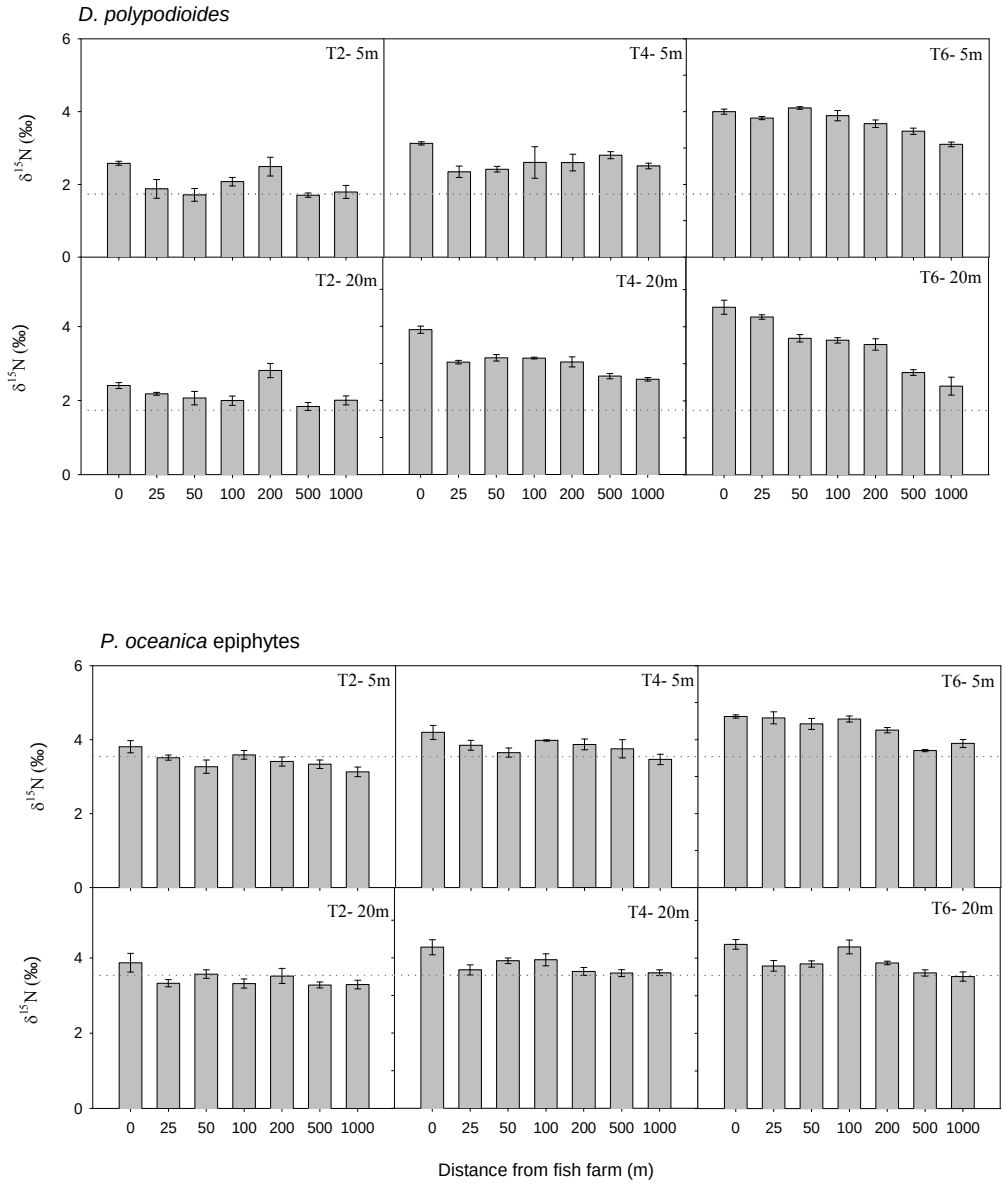


Figure 4: Nitrogen isotopic ratio ($\delta^{15}N$) in the two macroalgae analyzed in Murcia, *Dictyopteris polypodioides* and *Posidonia oceanica* epiphytes. For each specie of macroalgae the results for two depths (5-20m) and three times of incubations (T2= 2 days; T4= 4 days; T6= 6 days) at different distances from fish farm are shown. Vertical bars correspond to the standards error of the mean.

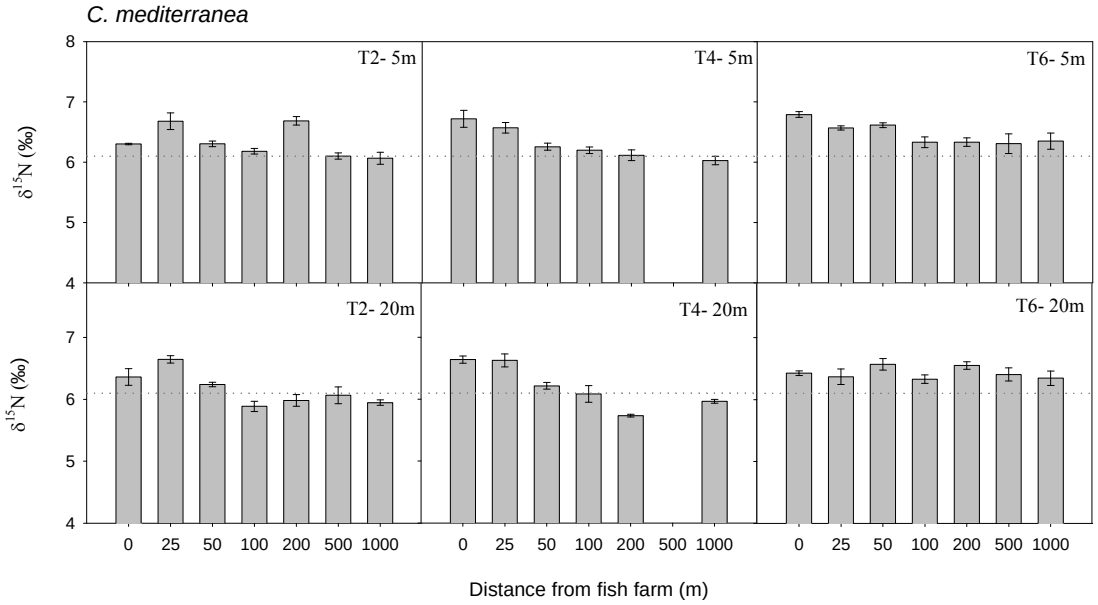


Figure 5: Nitrogen isotopic ratio ($\delta^{15}N$) in the macroalgae analyzed in Catalonia, *Cystoseira mediterranea*. The results for two depths (5-20m) and three times of incubations (T2= 2 days; T4= 4 days; T6= 6 days) at different distances from fish farm are shown. Vertical bars correspond to the standards error of the mean.

Total nitrogen (% N) content

The mean N content values obtained at T2 (2 days) were similar to the reference values (Figs. 6, 7 and 8 and Table 1) and there were no significant differences between transect distances, with two exceptions: *P. oceanica* epiphytes at a depth of 5 m and *C. mediterranea* at both 5 and 20 m depth (Table 3). However, in these cases no clear spatial trend associated with fish cages (Figs. 6, 7 and 8 and Table 3) was detected. In T4 (four days) differences between transect distances were found, such as in *A. taxiformis* at a depth of 5 m and in *D. polypodioides* and *C. mediterranea* at a depth of 20 m. From these cases, *D. polypodioides* at a depth of 20 m was the only species to show a perceptible spatial gradation. However, at T6 (6 days) significant

differences were found between transect distances in almost every species except *S. zonale* and *P. oceanica* epiphytes (at a depth of 20 m both); *D. polypodioides* and *P. oceanica* epiphytes followed significant trends at a depth of 5 m, while the same was true for *C. mediterranea* at a depth of 20 m (Figs. 6, 7 and 8 and Table 3). Differences between the fish cage (0 m) and control (1000 m) sites were greater in Murcia than in Catalonia (1.6-fold and 1.2-fold, respectively).

Total phosphorus (% P) content

Total P content (% P), which was only measured in *Cystoseira mediterranea* tissue, showed no significant differences ($p > 0.05$) between transect distances in T2 (2 days) in the two depths studied (Fig. 9 and Table 4). In T4 (4 days), there was significant variation between mean values along the transect. However, there was no clear tendency of increasing values near the fish farm with respect to the reference value (Table 1). In contrast, a gradual but significant increase of % P values respect to the reference value towards the fish farm was found after six days of incubation (T6). This increase was detected at both incubation depths (5 and 20 meters) (Fig. 9 and Table 4). The control and reference values were similar in the two cases.

Table 3 Summary of ANOVA one-way results for % N values of the macroalgae analyzed. *Post hoc* associations are shown in lower-case letters. For the sake of simplicity, only SNK test results for significant differences are shown. H_o: there are no significant effects between distances. NS = not significant, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

Site	Specie	Samples		ANOVA one-way (p-value)	SNK - test (distance)						
		Depth	Time		0	25	50	100	200	500	1000
Canary Islands	*Asparagopsis taxiformis*	5	T2	ns	-	-	-	-	-	-	-
			T4	** (1)	a	b	b	b	b	b	b
			T6	***	d	cd	cd	cd	a	b	c
		20	T2	ns	-	-	-	-	-	-	-
			T4	**	ab	bc	bc	bc	a	c	bc
			T6	**	cd	abc	abc	cd	ab	a	abc
	Stypopodium zonale	5	T2	ns	-	-	-	-	-	-	-
			T4	ns (1)	-	-	-	-	-	-	-
			T6	*	a	a	a	a	a	a	a
		20	T2	ns	-	-	-	-	-	-	-
			T4	ns	-	-	-	-	-	-	-
			T6	ns	-	-	-	-	-	-	-
Murcia	*Dictyopteris polypodioides*	5	T2	ns	-	-	-	-	-	-	-
			T4	ns (1)	-	-	-	-	-	-	-
			T6	**	a	ab	ab	a	ab	ab	b
		20	T2	ns	-	-	-	-	-	-	-
			T4	**	ab	ab	ab	a	a	ab	b
			T6	** (1)	abc	a	bc	ab	abc	c	c
	Posidonia oceanica epiphytes	5	T2	**	ab	ab	ab	a	ab	b	b
			T4	ns	-	-	-	-	-	-	-
			T6	***	a	ab	ab	bc	bc	c	c
		20	T2	ns	-	-	-	-	-	-	-
			T4	ns	-	-	-	-	-	-	-
			T6	ns	-	-	-	-	-	-	-
Catalonia	*Cystoseira mediterranea*	5	T2	*** (1)	b	a	b	b	b	b	b
			T4	* (1)	-	-	-	-	-	-	-
			T6	*	a	a	a	a	a	a	a
		20	T2	**	ab	a	ab	ab	b	ab	b
			T4	*	a	a	a	a	a	a	a
			T6	*** (1)	a	abc	ab	abc	c	bc	d

(1) Non homogeneous variances. Significant differences if p ≤ 0.01

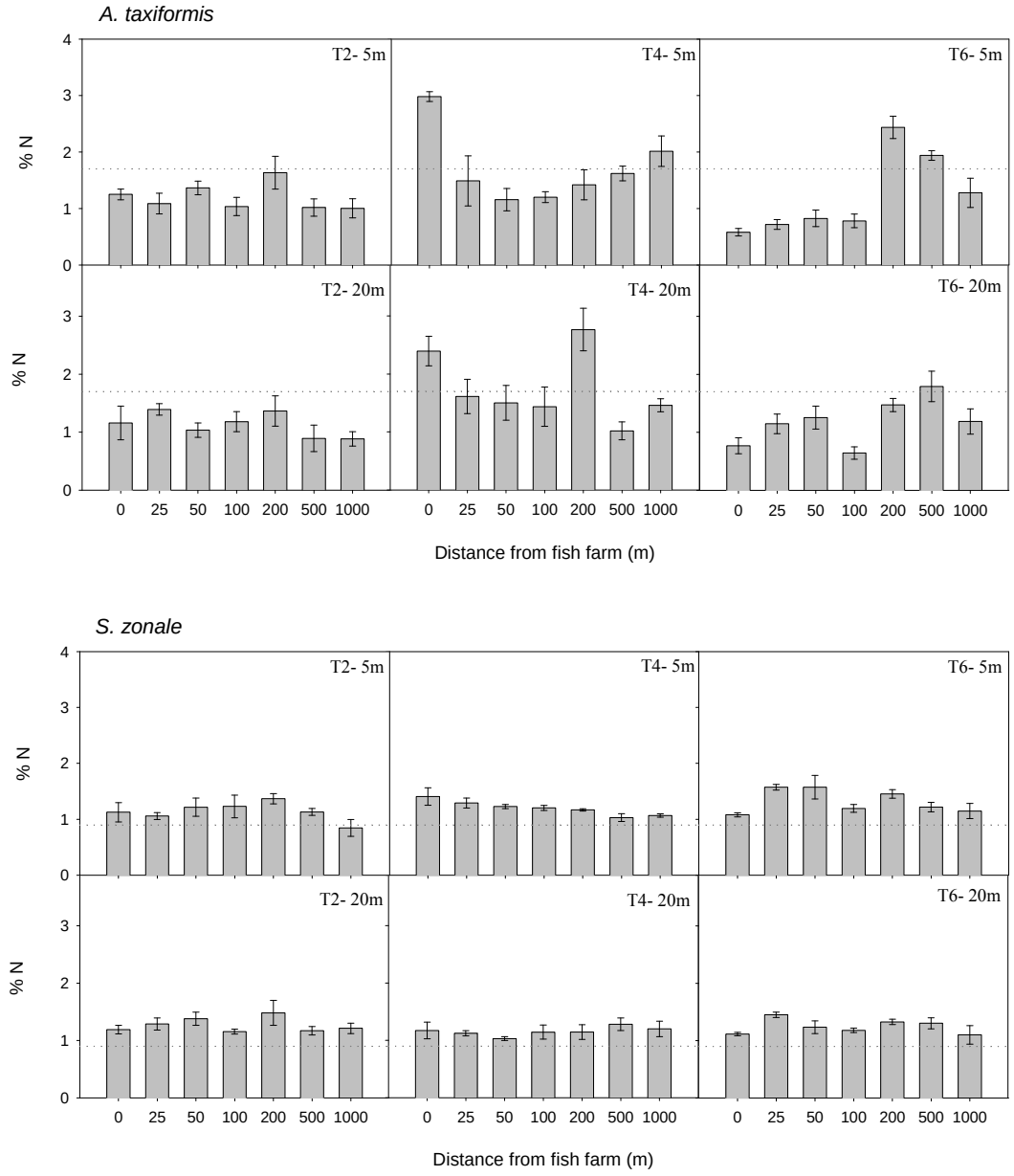


Figure 6: Nitrogen content (% N) in the two macroalgae analyzed in Canary Islands, *Asparagopsis taxiformis* and *Stypopodium zonale*. For each species of macroalgae the results for two depths (5-20m) and three times of incubations (T2= 2 days; T4= 4 days; T6= 6 days) at different distances from fish farm are shown. Vertical bars correspond to the standards error of the mean.

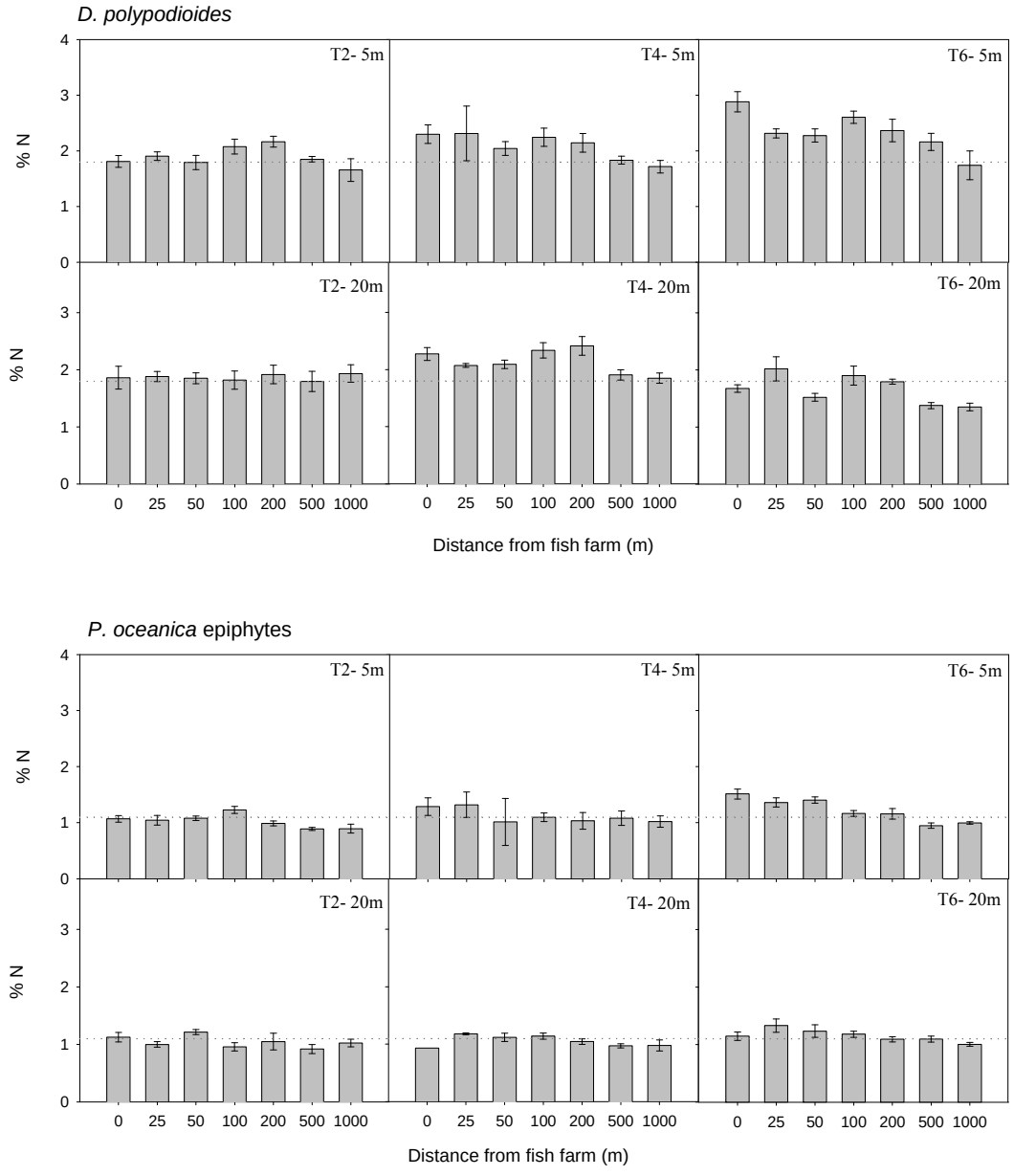


Figure 7: Nitrogen content (%N) in the two macroalgae analyzed in Murcia, *Dictyopteris polypodioides* and *Posidonia oceanica* epiphytes. For each species of macroalgae the results for two depths (5-20m) and three times of incubations (T2= 2 days; T4= 4 days; T6= 6 days) at different distances from fish farm are shown. Vertical bars correspond to the standards error of the mean.

Table 4 Summary of ANOVA one-way results for % P values of the macroalgae analyzed. *Post hoc* associations are shown in lower-case letters. For the sake of simplicity, only SNK test results for significant differences are shown. H_o: there are no significant effects between distances. NS = not significant, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

Site	Specie	Samples		ANOVA one-way (p-value)	SNK- test (distance)						
		Depth	Time		0	25	50	100	200	500	1000
Catalonia	*Cystoseira mediterranea*	5	T2	ns (1)	-	-	-	-	-	-	-
			T4	*	a	ab	b	ab	ab	ab	a
			T6	*	a	ab	ab	ab	b	ab	b
		20	T2	ns (1)	-	-	-	-	-	-	-
			T4	*	a	a	a	a	a	a	a
			T6	***	a	a	a	b	b	b	b

(1) Non homogeneous variances. Significant differences if p ≤ 0.01

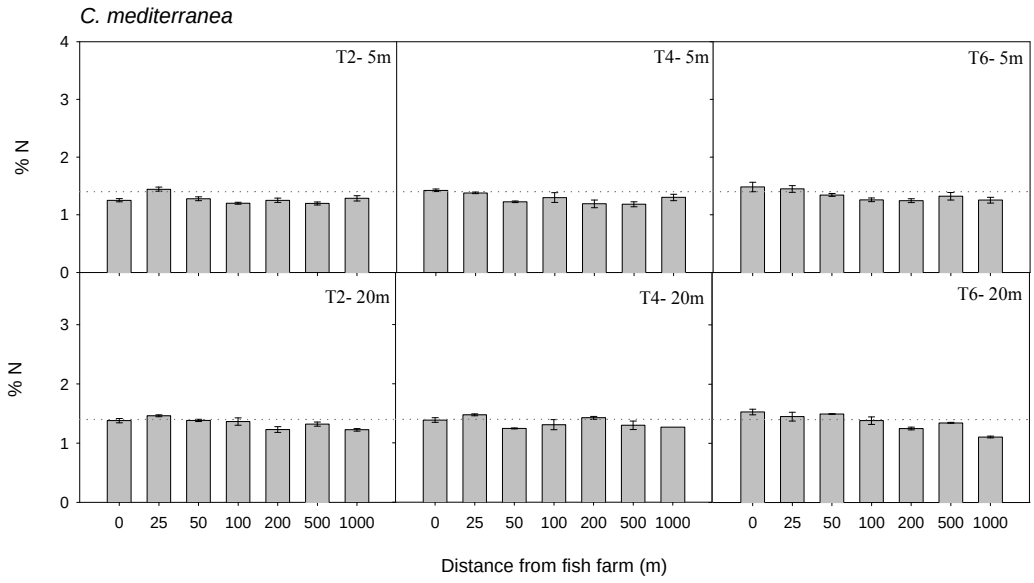


Figure 8: Nitrogen content (% N) in the macroalgae analyzed in Catalonia, *Cystoseira mediterranea*. The results for two depths (5-20m) and three times of incubations (T2= 2 days; T4= 4 days; T6= 6 days) at different distances from fish farm are shown. Vertical bars correspond to the standards error of the mean.

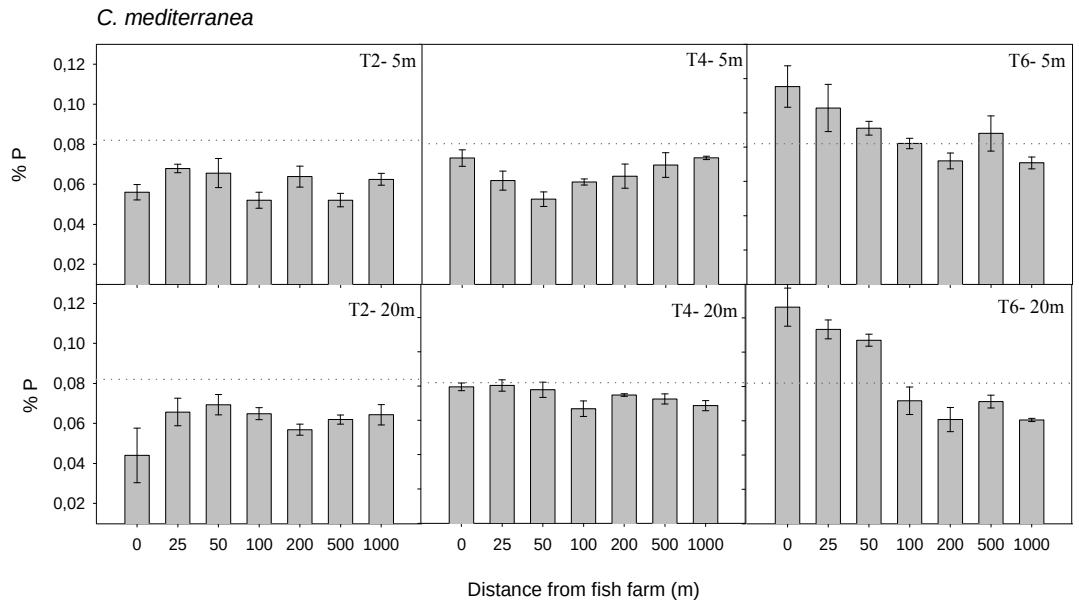


Figure 9: Phosphorus content (% P) in the macroalgae analyzed in Catalonia, *Cystoseira mediterranea*. The results for two depths (5-20m) and three times of incubations (T2= 2 days; T4= 4 days; T6= 6 days) at different distances from fish farm are shown. Vertical bars correspond to the standards error of the mean.

Discussion

In general terms, the results obtained in this study show a gradual and significant increase of the $\delta^{15}N$ values in incubated macroalgae towards the fish cages respect to the reference (initial) and control values, which confirm the validity of the bioassay method employed to detect and characterize spatial gradients of nutrients associated to offshore aquaculture facilities.

This response was similar to the increase in $\delta^{15}N$ in benthic primary producers of natural communities under the influence of organic effluents delivered from sewage outfalls

(Costanzo et al., 2001) or from land-based aquaculture facilities (Jones et al., 2001, Vizzini and Mazzola, 2004). Contamination from animal wastes is a common cause of ^{15}N enrichment in aquatic environments due to isotopic fractionation during the ammonification and nitrification processes of urea, which is one of the main forms of excreted nitrogen (Macko and Ostrom, 1994). Thus, higher $\delta^{15}N$ values in incubated macroalgae indicate a significant assimilation of dissolved inorganic nitrogen forms (ammonia and nitrates) coming from fish farm wastes. Therefore, decreasing $\delta^{15}N$ signature in incubated macrophytes with increasing distance from the fish cages is likely to reflect the dilution of nutrients from the origin. This agrees with the high significant negative correlation that we have found between the isotopic signature and the distance from the fish cage for most of the case studies (Table 2).

The spatial pattern found for $\delta^{15}N$ was well-defined and significant after four days of incubation (T4) (Table 2). After two days of bioassay deployment (T2), differences in $\delta^{15}N$ were not significant and did not show any consistent spatial pattern. In contrast, in T4 (four days) and T6 (six days) well-defined gradients were found. Then, although nutrients released from fish farms usually show large temporal variability (e.g. Karakassis et al., 2001), an incubation time of 4-6 days was sufficient to integrate temporal, short-term changes in the nutrient regime at increasing distances from fish farms. These findings are consistent with results from other studies where macroalgae assimilated sewage-derived DIN and displayed higher $\delta^{15}N$ values in their tissues at similar times of incubations (Gartner et al., 2002; Costanzo et al., 2004; Deutsch and Voss, 2006).

Not all of the macroalgal species tested showed the same tolerance to survive to the experimental procedure of the bioassays. The red algae *A. taxiformis* used in the bioassays at the Canary Islands site was the most obvious case (Fig. 3). In this specie, values of both $\delta^{15}N$ and total N content along transects did not show any relationship with the fish farm effluent. Indeed, $\delta^{15}N$ and total N content strongly decreased in relation to references values (Tables 2 and 3). This is explained by tissue decomposition during the bioassay incubation and indicates the low

resistance of this macrophyte to experimental manipulation. The other macroalgae species and *P. oceanica* epiphytes were resistant to manipulation as no signs of deterioration were apparent during incubation and we assumed that they were assimilating nitrogen derived from fish-farm, so they could be considered as good indicators of nitrogen derived from fish farms wastes.

Spatial gradients between the fish farm and control sites were more clearly defined in the upper layer of the water column (i.e. 5 m incubation depth) than at a depth of 20 m, where this gradient was not well-defined (e.g. *P. oceanica* epiphytes; Fig. 4) or inexistent (e.g. *S. zonale*; Fig. 3). This can be attributed to differences in environmental factors as light (Dudley, 2007) or differences in the dispersion patterns of wastes. Macroalgae incubated at 20 m are subjected to lower light levels than macroalgae incubated at 5 m. This can affect the photosynthetic rates and hence nitrogen assimilation (Lobban and Harrison 1997). On the other hand, our results indicated a higher dispersion of the dissolved inorganic nitrogen forms derived from fish-farm wastes in the superficial layer than in the deeper one. This can be attributed to a high fish density and activity in the upper layer during feed operations. Also, in summer, a thermocline was established between the two incubation depths (unpublished data, pers. obs), which prevented effective diffusion and transport of dissolved nutrients towards the deeper layer, so fish farm wastes are more easily dispersed, and thus detected by the bioassays in the superficial layer.

The magnitude of $\delta^{15}N$ enrichment of macrophyte tissues (in relation to reference, initial and control mean values) appeared higher also in the superficial layer than in the deeper one in all study sites, except in Murcia for *D. polypodioides*. In Murcia study, results show that the dispersion of the fish farm effluent in the superficial and in the deeper layer reached further than the scale measured in this study (1000 m), especially in the superficial layer, since control bioassays had an isotopic signal higher than these found in reference algae. This is consistent with the larger size of the aquaculture facilities selected in Murcia (see methods), where the

annual fish production is 9- and 20-fold higher than in the Catalonia and Canary Islands fish farms, respectively.

The sensitivity of the bioassay and its spatial variability may be also influenced by other factors such as the nutrient regime characteristics of each coastal area and the presence of nutrient sources that differ from those derived from offshore fish farms (Ye et al., 1991; Wu, 1995; Cromey et al., 2002; Vizzini and Mazzola, 2004; Sarà et al., 2006). These factors varied between the three fish farms studied here, which is probably reflected in the results. The variation of $\delta^{15}N$ along the transect was higher in the fish farms of Murcia and the Canary Islands (2.5 -5 ‰), probably due to the oligotrophic character of their waters. This condition, together with the absence of any substantial source of nitrogen in the studied areas, besides fish farms, meant that $\delta^{15}N$ could be applied as a single tracer to follow the dispersion of the fish farm wastes from source point (Peterson, 1999). In contrast, the variations of $\delta^{15}N$ in Catalonia were lower than those in Murcia and the Canary Islands, mainly because of higher initial $\delta^{15}N$ values in natural stands of *C. mediterranea* (6-7 ‰) used as reference values. The $\delta^{15}N$ values found in *C. mediterranea* in our study site are clearly higher that the background values (3.4 ‰, Pantoja et al., 2002) for dissolved N in the Western Mediterranean and that those found in the genus *Cystoseira* from unpolluted environments (Pinnegar and Polunim, 2000). This could be explained by the high continental influence of the Catalonia fish farm. Coastal waters near continents are enriched in the heavier N isotope transported by runoff from watersheds larger than in islands, and with important urban and agricultural development (McClelland and Valiela, 1998; Bowen and Valiela, 2008). Consequently, the contribution of fish farm waste to the enhancement of $\delta^{15}N$ in incubated algae could have been masked by ambient levels. In these cases the use of the nitrogen stable isotope ratios would not have been as sensitive as in oligotrophic areas to detect dissolved nutrients derived from fish farm (Costanzo et al., 2003); to be more effective it could be accompanied by other macrophyte indicators such as total phosphorus (% P), which is abundant (although in a lesser extent than nitrogen) in fish farm wastes (Holmer et al., 2008). Indeed, in the Catalonia study case the analysis of total P in the

incubated macrophytes revealed an intense and significant spatial gradient between 0 and 100 m. Nonetheless, these results contrast with those found by Dalsgaard and Krause-Jensen (2006) where the content of N, but not of P content, in *Ulva* sp. tissue was highest closest to the cages.

Total N content in marine plants is commonly considered a potential indicator of the concentration of biologically available nutrients in the environment (Duarte, 1990; Fong et al., 2004). Despite this, it was much less sensitive to detect fish farm waste than $\delta^{15}N$ probably due to high dispersion rates in offshore fish farms. In our study, total N content was a poor indicator of nutrient release from fish farms; in only three cases % N content increased towards the cages with respect to the reference and control values (*D. polypodioides* and *P. oceanica* epiphytes at 5 m and *C. mediterranea* at 20 m; Table 4). This trend was also reported by Lin and Fong (2008), who reported a much more localized response to nutrient availability in tissue N content than in nitrogen isotope stable ratios in relation to shrimp farm effluents. These authors attributed this response to the fact that nutrient supply was not high enough to induce tissue nitrogen storage (Lin and Fong, 2008), which only occurs when nutrient supply exceeds growth rate (Fong et al., 2004). In our study, the lack of response in tissue nitrogen content could be explained by the same argument; it is likely that the high hydrodynamic conditions in offshore fish farms dilute the nitrogen rapidly. Moreover, the lack of nitrogen response may also be influenced by other aspects related to species-specific physiological traits (Fong et al., 2001, 2003).

In summary, our results confirm the potential of $\delta^{15}N$ in macrophytes (more than the classic measures of total N and P tissue content) to track the dispersion of dissolved nutrients derived from aquaculture discharges in offshore sites without benthic vegetation, and especially in oligotrophic waters. Changes in $\delta^{15}N$ values measured in incubated macrophytes along transects has provided clear and reliable characterizations of nutrient gradients associated with the fish farm wastes in 4-6 days (except for *A. taxiformis*), and the wastes are going farther in the upper layer of the water column than in the deeper. Furthermore, the technique is simple and

low cost; experimental deployment is very easy to perform and requires very little training making this method a strong candidate for application in monitoring programs.

Finally, our results have also highlighted the necessity of an assessment study to determine the experimental settings (species of macroalgae, distances from fish farms, incubation depth, etc…) that will ensure the bioassay is successful in indicating the area of the spatial extent of fish farm wastes. The results obtained in this study now make it possible to extensively apply the bioassay to the case studies. A proper spatial replication is able to gather most of the spatial variability caused by currents and other local factors of interest i.e. more than one site per distance and at least two directions. In conclusion, the application of this macroalgal bioassays method have a great potential in determining the spatial scale of the influence of aquaculture wastes on the marine environment, which represents a key aspect for monitoring and management this activity in coastal marine ecosystems.

Chapter **4**

Assessment of the spatial extent of offshore fish-farm waste using the nitrogen stable isotope ratio ($\delta^{15}N$) in macroalgal bioassays

Evaluación de la extensión espacial de vertidos de granjas marinas usando el isótopo estable del nitrógeno ($\delta^{15}N$) en bioensayos con macroalgas

Resumen

En este estudio se ha evaluado la dispersion de los vertidos procedentes de granjas marinas *offshore* mediante el análisis de los ratios del isótopo estable del nitrógeno ($\delta^{15}N$) en macroalgas incubadas a distancias crecientes de las jaulas marinas. Los bioensayos se dispusieron en tres granjas marinas situadas en localidades separadas con diferentes condiciones nutricionales (Islas Canarias, Murcia y Cataluña) y con diferencias en el tamaño, especie cultivada y producción anual. Los bioensayos de macroalgas fueron situados a lo largo de dos direcciones (DI y DII) y fueron replicados en cada distancia con el fin de evaluar el efecto de la variabilidad a pequeña escala en la extensión espacial de los vertidos de granjas marinas. Los resultados obtenidos con el análisis de $\delta^{15}N$ contribuyen a incrementar la información sobre la aplicación de los isótopos estables del nitrógeno en macroalgas como un indicador efectivo para trazar la dispersión de nutrientes procedentes de granjas marinas offshore y demuestra que los vertidos de las granjas marinas pueden ser detectados a distancias de hasta ut the application of nitrogen stable isotopes ratios in macroalgae as an effective bioindicator to trace the dispersion varios kilómetros de la fuente de polución. En las Islas Canarias, la distancia de detección de los vertidos de las granjas mmarinas fue de 450-700 m. En Murcia encontramos la mayor distancia de influencia de los vertidos de granlas marinas de last res instalaciones estudiadas, estando entre 1550-2450 m y en Cataluña la distancia encontrada fue de menos de 120 m. En Cataluña, estos resultados fueron enmascarados por la influecia de otra fuente de nitrógeno, obteniendo distancias de detección menores de las esperadas en función de la producción de esta granja. Estos resultados confirman que los vertidos procedentes de granjas marinas pueden ser trazados usando loslos isótopos

estables de N lo que puede resultar útil para identificar areas de riesgo potencial para ecosistemas sensibles o ser un indicador temprano de cambios que podrían ocurrir en la estructura de las comunidades.

Abstract

In this study, the dispersal of wastes loaded from offshore fish farms were evaluated analyzing nitrogen stable isotope ratios ($\delta^{15}N$) in macroalgae incubated in the water column at sites increasingly further from the fish cages. Bioassays were performed in three fish farms placed in separated localities with different nutritional conditions (Canary Islands, Murcia and Catalonia) and with differences between them in size, specie of fish reared and annual production. Macroalgal bioassays were placed along two different directions (DI and DII) and they were replicated at each distance to evaluate the effect of small-scale variability on the spatial extent of fish-farm wastes. The results obtained with $\delta^{15}N$ contribute to increase the information about the application of nitrogen stable isotopes ratios in macroalgae as an effective bioindicator to trace the dispersion of offshore fish-farm wastes and demonstrate that fish farm wastes can be traced even over distances of some km from the pollution source. In Canary Islands the distance of detection of fish-farm wastes obtained was of 450-700 m. In Murcia we found the highest distance of influence of fish farm wastes of the three installations studied, ranging between 1550-2450 m and in Catalonia this distance was of less of 120 m. In Catalonia, the results were masked by the influence of other source of nitrogen, obtaining distances of detection of fish-farm wastes smaller than expected. These results confirm that fish-farm wastes could be traced using the nitrogen stable isotope ratios of macroalgae and it can be useful to identify the areas of potential risk for some sensitive ecosystems or be an early signal that changes in the community structure might occur.

Keywords: Nitrogen Stable isotopes ($\delta^{15}N$), Fish farms, Macroalgae, Bioassays.

Introduction

While the world's population continues to grow, the supply of seafood products from marine capture fisheries may be reaching its limit. By this reason, aquaculture continues to grow more rapidly than all other animal food-producing (GESAMP, 2008). Specifically, intensive aquaculture in floating cages is expanding in off-shore coastal areas of many countries due to limitations on near-shore water space for this emergent industry (FAO, 2006).

Negative effects of this activity on coastal marine ecosystems are related to the high concentrations of suspended solids and dissolved substances release by the aquaculture operations that represent a significant nutrient loading to the surrounding environment (Lefebvre et al., 2001; Nordvarg and Johansson, 2002; Islam, 2005; Roque d´Orbcastel et al., 2008). The dispersal of these nutrients in receiving waters varies in function of a large number of factors related to the fish farm facility (farm size, amount of cultivated biomass, cultivated species and feed type, among others), to environment features (currents, local hydrodynamic regimen, depth, water physicochemical properties, sediment type, etc..), to ecosystem type as well as to the contextual presence of other anthropogenic disturbances (Wu, 1995; Vizzini and Mazzola, 2004; Sarà et al., 2006; Holmer et al., 2008). So, the development of effective methods able to characterize the dispersal of nutrients integrating the variability at different local conditions and spatial scales are required to proper control and management aquaculture activities and to aid to preserve the integrity of coastal ecosystems.

Standard physico-chemical analyses of seawater nutrient concentrations have been systematically used in monitoring programs to document the release of nutrients derived from aquaculture in the water column (McGhie et al., 2000; Jones et al., 2001; Burford et al., 2003; Sarà et al., 2004; Holmer et al., 2008). However these methods are ineffective since nutrient loads are rapidly diluted by hydrodynamic forces and/or removed by microbial and plant uptake, and also are labour-intensive and expensive. In effect, water quality sampling has shown

not to be enough sensitive to proper characterization of spatial gradients of nutrients released from off-shore fish farms as it has been evidenced by Sarà (2007) in a recent review. Practically in all of the studies performed in marine open waters there was no change or only a localized increase in water column nutrients concentrations close to the fish farm net-cages. This is attributed to the high dispersal of fish-farm waste and its extremely high spatio-temporal variability in these open water environments (Samocha and Lawrence, 1997; Karakassis et al., 2001; Vezzulli et al., 2008).

Benthic macrophytes have demonstrated to be more reliable indicators of the effects of anthropogenic nutrient loads in aquatic ecosystems. Marine plants uptake and assimilate nutrients in excess from the water column and accumulate them in their tissues, acting as reliable long term time-integrators of both continuous and pulsed nutrient loadings (e.g. Cohen and Fong, 2005; Cole et al., 2005; Jones et al., 2001). Nonetheless, the application of macroalgae bioindicators is limited by the heterogeneous distribution of natural communities in each locality. The use of primary producers (phytoplankton and macroalgae) bioassays as bioindicators of nutrient availability has been proposed as an alternative method to overcome such problem and has demonstrated to be very effective in the characterization of continuous nutrient gradients created by anthropogenic sources in coastal areas (e.g. Dalsgaard and Krause-Jensen, 2006; Deutsch and Voss 2006; Costanzo et al., 2001). By other part, the analysis of nitrogen stable isotopes in plant tissues has also appeared as another promising tool to trace wastes from specific sources in coastal ecosystems (Lepoint et al., 2004; Cole et al., 2004, 2005). Animal wastes are enriched in the heavy nitrogen isotope (N^{15}) compared to natural nitrogen sources (Lahtja and Michener, 1994). The isotopic N signal measured in marine benthic macrophytes has been shown to increase as they uptake nitrogen from aquaculture wastes (Jones et al., 2001; Costanzo et al., 2003, 2004), even in situations of low nutrient concentrations unable to cause changes in the total plant N content (Lin and Fong, 2008). In fact, isotopic analysis of natural macrophyte communities surrounding fish farms has evidenced

that the influence area of aquaculture wastes can be much larger than previously thought (Vizzini and Mazzola, 2004, 2006; Ruiz et al., in press).

Thus, the combination of macroalgal bioassays with nitrogen stable isotopes analysis point to be a more powerful, specific and reliable approach to characterize the spatial pattern and scales of dispersal of dissolved nutrients delivered from specific anthropogenic sources. The effectiveness of this approach has been evaluated in only few occasions to assess the extent of dissolved nutrients derived from urban (Costanzo et al., 2001; Deutsch and Voss, 2006) and land-based shrimp farms (Lin and Fong, 2008) wastes in near-shore coastal areas. In the present study we use by first time a technique involving the analysis of stable isotopes ratios ($\delta^{15}N$) in macroalgae bioassays to asses the spatial extent of aquaculture effluents delivered from floating cages concentrated in off-shore waters of many coastal areas. To this end, $\delta^{15}N$ values was measured in macroalgae from unpolluted sites incubated in replicated bioassays deployed at increasing distances from fish cages. The experiment was performed in three fish farms, two in the Mediterranean Sea (Catalonia and Murcia) and one in the Atlantic (Canary Islands). Additional measurements of total N content were performed in the incubated macrophytes to assess if N was arriving in excess to the plant. The results obtained in this study are analyzed and discussed in order to provide reliable estimates of the extent of the dispersal area of the aquaculture wastes in the coastal environments, meaningful information requested by coastal planners to manage aquaculture development and reduce its environmental risk on sensitive habitats.

Materials and Methods

Study sites

The study was performed in three offshore fish farms located in warm-temperate areas of the Spanish coast. One was located in the Atlantic Ocean, in the coast of Tenerife Island (Canary Islands) and two in the western Mediterranean Sea (Murcia and Tarragona -Catalonia coast-) (Fig. 1). In the Canary Islands fish farm, the annual production during the year of study was about 375 tons. The species farmed were sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). Murcia fish farm produced about 350 tons of Atlantic blue fin tuna (*Thunnus thynnus*). This facility was at the southern end of a larger complex of seven fish farms concentrated in an area of 1.47 km^2 and with a total annual production of 6,197 tons of blue fin tuna, sea bream and sea bass. In Catalonia, the annual production of the fish farm was about 800 tons of sea bream. This farm was located northward the delta of the Ebro River. All fish farms selected were located in soft, unvegetated bottoms between 20 and 40 m deep. Besides the fish farm, no other local source of pollution was present in the selected study sites. The study was carried out between July and August in 2006, a season of the year (summer) in which light, temperature and fish production are at its maximum in all locations. One experiment was performed in each fish farm, except in Murcia, where the experiment was performed twice (i.e. in two consecutive periods) to assess temporal variability of the spatial patterns analyzed.

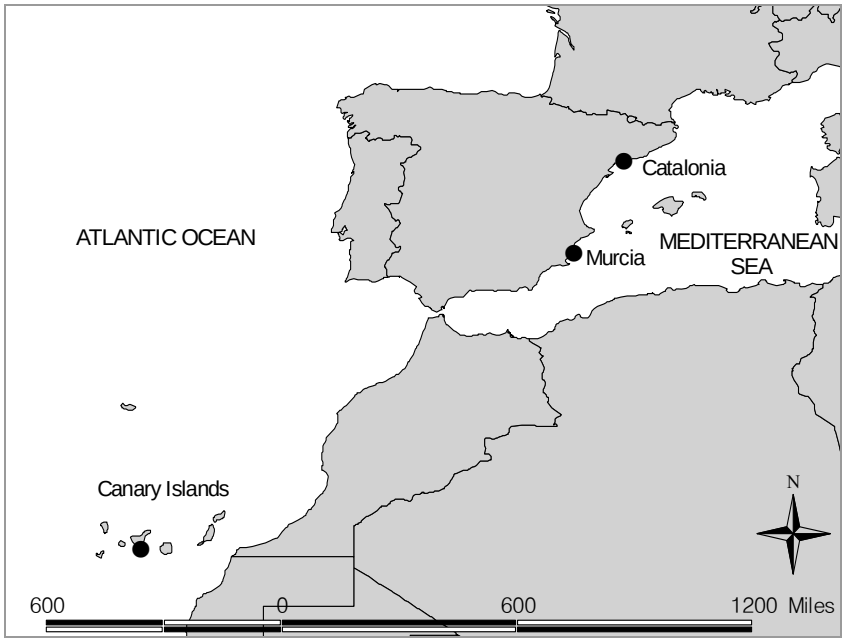


Figure 1: Sample sites map.

Experimental design

The settings of the bioassay parameters (i.e. algae species, incubation depth and time and distances from fish cages) were selected from a previous pilot study performed one year before at the same study cases (García-Sanz et al., in press). At each study site, the day prior to the bioassay deployment, macroalgae samples were collected from natural algae communities of nearby unpolluted sites. *Stypopodium zonale* (Lamouroux) was used in the Canary Islands study site, *Dyctiopteris polypodioides* (De Candolle) was used in Murcia fish farm and *Cystoseira mediterranea* (Sauvageau) was used in Catalonia fish farm. Macroalgae were maintained in coolers with well-aerated seawater under low light conditions to minimize physiological stress by changes in light and temperature. Ten samples were separated from the bulk of collected plant material and keep frozen until the time of analysis to determine natural values of the variables analyzed (i.e. reference values).

At each fish farm we choose two different directions in order to assess spatial anisotropy of nutrient gradients around fish farm due to hydrodynamic factors: one parallel to the coastline (DI) and other perpendicular (DII), toward the coast (Fig. 2).

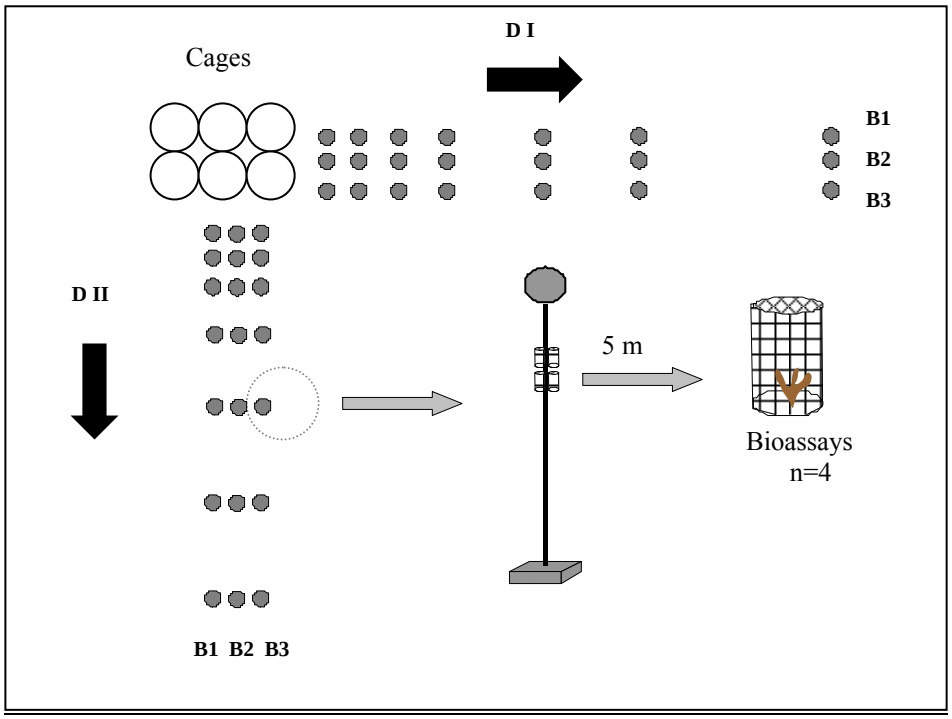


Figure 2: Diagram of the experimental design developed in this study.

The direction of D1 was selected for each case based on the dominant current direction established from data available in their respective monitoring programs. At each direction, bioassays were deployed at increasing distances from fish cages, being the largest distance considered as a control site representative of a natural, unpolluted condition. The set of distances was selected for each study case (Table 1) accordingly with the information obtained in the previous pilot study (García-Sanz et al., in press). In the Murcia study case, and based in

this and other previous study (Ruiz et al., in press), we were obligated to establish the control site in a marine protected area located 14 km southward; the large spatial scale of wastes derived from this large fish farm complex and the possible influence of other pollution sources (Ruiz et al., in press) justify this decision. The same control site was used to analyze nutrient gradients in DI and DII directions.

Table 1 Distances of macroalgal bioassays from the fish farm cages at each study site along two different directions (DI and DII).

Fish farm	Distances from fish farm (m)						
Canary Islands	0	25	50	100	200	500	1000
Murcia	0	75	260	520	1100	2700	15000
Catalonia	0	50	100	200	500	DI: 1000 DII: 800	DI: 2000 DII: -----

For each direction, three buoys (B1, B2 and B3) combined with a rope and a weight, were installed within each distance in three aligned sites separated 25 m among them as represented in Figure 2. A bioassay unit consisted in a piece of macroalgae (25-50 g fresh weight) housed into a incubation chamber made of 1 cm plastic mesh cylinders of 9 cm diameter and 15 cm height (Fig. 2). For each site, four of these units were attached to the rope of each buoy at a depth of 5 m from the sea surface and collected after 4-6 days (García-Sanz et al., in press). This represent a nested sampling design (Quinn and Kenough 2002) used to partition the spatial variability of the measured variables ($\delta^{15}N$ and %N) into small-scale (i.e. between sites within each distance) and large-scale (i.e. across distances) variability. Plant material contained in each incubation device was cleaned with deionized water, stored in labelled plastic bags and kept frozen until analysis.

Analysis of plant material

All macroalgae samples were dried at 60ºC to constant weight for 24 hours, ground using an agate mortar and pestle and preserved in a desiccator at room temperature. From each sample, between 1.8 and 2 mg of dry weight was encapsulated to determine total nitrogen content (% N) and the stable isotope ratio ($\delta^{15}N$).

The isotopic analysis technique was based on the two naturally occurring atomic forms of nitrogen ^{14}N and ^{15}N (Mariotti, 1983). By measuring the ratio of ^{14}N and ^{15}N in dried plant tissue, the relative amount of ^{15}N or $\delta^{15}N$ in the plant can be determined as the relative difference between the sample and a worldwide standard (atmospheric N_2) using the following equation (Peterson and Fry, 1987):

$$\delta^{15}N = (^{15}N/^{14}N_{sample} / {}^{15}N/^{14}N_{standard} - 1) \times 1000$$

The nitrogen stable isotope ratio and total N content were determined using a continuous flow isotope ratio mass spectrometer (Thermo Finnigan Delta Plus IRMS). This was carried out after combustion in an elemental analyzer Flash EA from TermoFinnigan coupled via a ConFlo II from Finnigan MAT with the elemental analyzer. The ratio $\delta^{15}N$ value was expressed in per mil (‰). The internal laboratory standard employed was acetanilide. $\delta^{15}N$ of particulate fish farm effluents (faeces, fish feed) were also analyzed.

Statistical procedures

At each study site and for each direction, a nested ANOVA model was used to test the null hypothesis that $\delta^{15}N$ and % N in plant tissues (dependent variables) were similar between and

within distances. A two factor model was considered being "distance" a fixed factor with seven levels (defined in Table 1) and "site" (buoy) a random factor nested in "distance" with three levels (B1, B2 and B3), with the bioassays units (n=4) as the residual error. Before analysis, Cochran's C-test was used to assess the homogeneity of variances and data were transformed when necessary. Differences were considered significant if $p<0.05$. When variances did not meet the assumption of homocedasticity, a probability level of 0.01 was used to reduce the possibility of a Type I error (Underwood, 1997). A post-hoc Student-Newman-Keuls (SNK) test was used to compare means values after significant effects in ANOVA was detected. In addition, variance components of the ANOVA model were calculated (Quinn and Keough 2002) for qualitative comparisons of spatial patterns. Statgraphics Plus 5.1 data analysis software package was used for the statistical analysis.

Spatial patterns of $\delta^{15}N$ were analyzed also to determine the maximum distance at which fish farm wastes are dispersed from fish cages. To this end mean values of this variable obtained in each distance were normalized to the mean of the control site (i.e. the largest distance considered in each study case) and expressed in relative units as a net increment of this value. By definition this normalized variable ranges between 0 in the control distance and 1 in the fish cage. Spatial patterns of $\delta^{15}N$ were then described by fitting, using least square regression analyses, a negative exponential equation to the relationship between distance and the relative net increment of this variable. This equation was selected since it produced the best adjustable model to the spatial patterns obtained. The maximum distance of fish farm dispersal was calculated from each equation by considering a relative net increment of $\delta^{15}N$ equal to 0.2. We consider this value as a threshold above which fish farm wastes cause an effect on this variable clearly differentiated from that caused by natural variability, measured in natural macroalgae populations (i.e. reference values in this study). Regression analyses were performed using the non-linear curve fitter module of the software package SigmaPlot 10.0.

Results

Nitrogen stable isotope ratios ($\delta^{15}N$)

Nitrogen stable isotope ratios ($\delta^{15}N$) in the macroalgal bioassays deployed at control sites showed similar values to that obtained in the reference samples (p-value>0.05; Table 2). On the other hand, the analysis of $\delta^{15}N$ in macroalgal bioassays deployed at different distances from fish farms showed a significant increase towards the cages in all fish farms studied (Fig. 3 and Table 3).

Table 2 Measurements of $\delta^{15}N$ and % N in macroalgae collected from unpolluted sites at each study site.

Fish farm	Specie	Reference values	
		$\delta^{15}N$ (‰)	Total N (%)
Canary Islands	*Stypopodium zonale*	2.7 ± 0.06	1.5 ± 0.05
Murcia	*Dyctiopteris polypodioides*	2.8 ± 0.02	2.0 ± 0.05
Catalonia	*Cystoseira mediterranea*	5.9 ± 0.13	1.5 ± 0.07

At the Canary Islands fish farm, mean $\delta^{15}N$ values of *Stypopodium zonale* increased from 2.7 ± 0.07 ‰ at control sites to 3.4 ± 0.06 ‰ at sites closest to the farm in the direction DI and from 3.2 ± 0.06 to 3.5 ± 0.05 ‰ in the direction DII. Mean values were significantly higher than control means up to the 500 m distance for DI and 25 m distance for DII (SNK, p=0.05; Fig. 3). The distance from fish farm accounted for a 36.4 to 53.2 % of the total variation, but the relative contribution of the variability between bioassays (i.e. error term) was equally important (46.8-55.1 %) (Table 3). In the Murcia study case, mean $\delta^{15}N$ values of *Dyctiopteris polypodioides* obtained in the first experiment (Murcia A) increased from 3.0 ± 0.05 ‰ at

control sites to 4.1 ± 0.07 ‰ near the farm in the direction DI and from 3.0 ± 0.05 to 3.9 ± 0.05 ‰ in the direction DII. For both directions, mean values were maintained higher than control means up to the 2,700 m-distance, although such difference was more clear for D1 than for DII (SNK, p=0.05; Fig. 3). In the second experiment (Murcia B) mean $\delta^{15}N$ values increased from 2.7 ± 0.06 ‰ at control sites to 4.1 ± 0.08 ‰ near the farm in the direction DI and from 2.7 ± 0.06 to 4.1 ± 0.07 ‰ in the direction DII. In this experiment, mean values were significantly and clearly higher than control means up to 1,100 m in DI and 2,700 m in DII (SNK, p=0.05; Fig. 3). In this study case, variability of $\delta^{15}N$ values in macroalgal bioassays were mainly accounted by the distance from fish farm (71.2-83.7 %; Table 3). In the Catalonia study case, $\delta^{15}N$ values of *Cystoseira mediterranea* increased from 5.9 ± 0.07 ‰ at control sites to 6.5 ± 0.08 ‰ near the fish farm in the direction DI and from 5.9 ± 0.07 to 6.4 ± 0.06 ‰ in the direction DII. Mean values were significantly and clearly higher than control means up to the 50 m-distance for both directions (SNK, p=0.05; Fig. 3). The component of the variance was similar for all levels considered in the ANOVA model (25.3-37.7 %; Table 3).

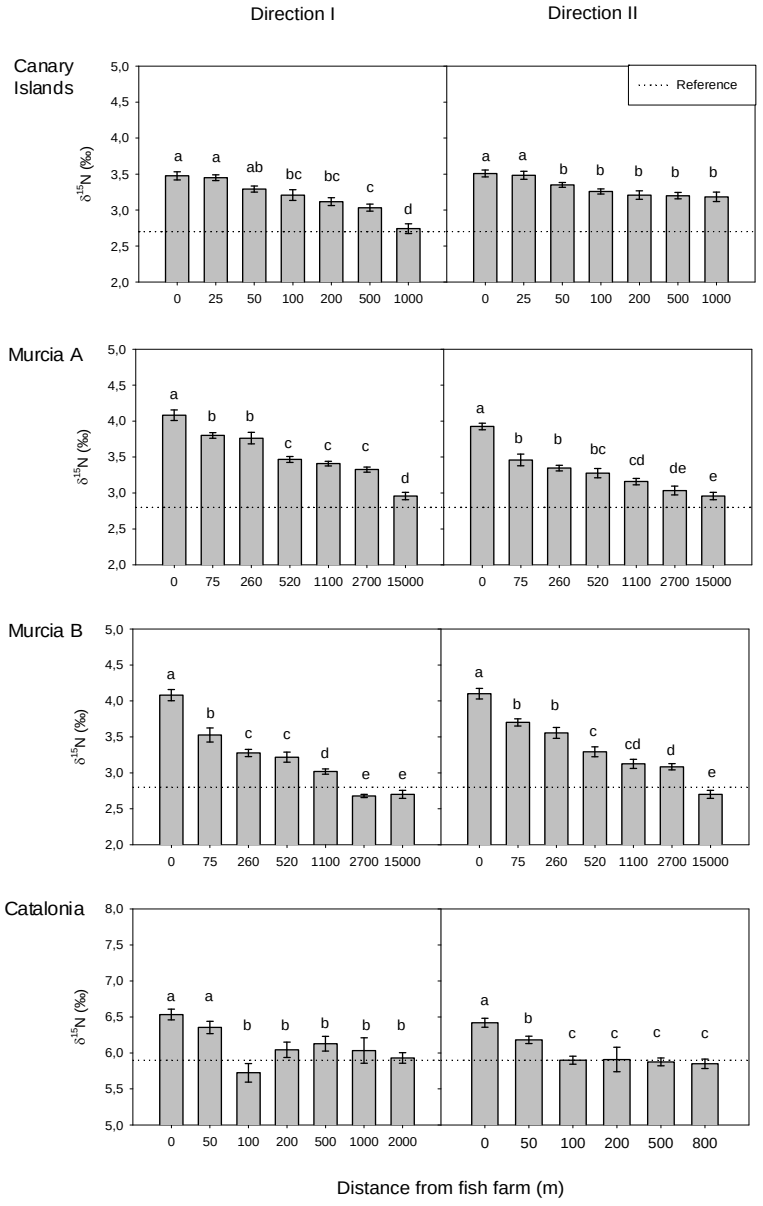


Figure 3: Nitrogen isotopic ratio ($\delta^{15}N$) in macroalgae incubated at different distances from fish farm in the Canary Islands (*Stypopodium zonale)*, Murcia (*Dyctiopteris polypodioides)* and Catalonia (*Cystoseira mediterranea*). For each species of macroalgae the results for two directions (DI and DII) are shown. For Murcia the results of two identical but separated in the time experiments are presented (Murcia A and Murcia B). Vertical bars correspond to the standards error of the mean.

Table 3 Summary of results of a nested ANOVA and estimated variance component (% var.) on nitrogen stable isotope ratios ($\delta^{15}N$) in macroalgae tissues incubated in three different study sites at different distances from fish farm. Each distance was replicated three times along two different directions (DI and DII). For Murcia the results of two identical but separated in the time experiments are presented (Murcia A and Murcia B). * p< 0.05; ** p < 0.01; *** p<0.001; ns= not significant.

	Canary Islands Direction I				Direction II			
	df	MS	% var.	*p*	df	MS	% var.	*p*
distance	6	0.78	53.2	***	6	0.24	36.4	***
site (distance)	14	0.02	0.0	ns	14	0.04	8.5	ns
Error	63	0.05	46.8		63	0.03	55.1	
	Murcia A Direction I				**Direction II**			
	df	MS	% var.	*p*	df	MS	% var.	*p*
distance	6	1.57	81.8	***	6	1.25	71.2	***
site (distance)	13	0.05	3.2	ns	14	0.05	3.4	ns
Error	59	0.02	15.1		62	0.04	25.4	
	Murcia B Direction I				**Direction II**			
	df	MS	% var.	*p*	df	MS	% var.	*p*
distance	6	2.40	83.7	***	6	2.40	81.9	***
site (distance)	13	0.07	3.9	*	11	0.11	6.7	**
Error	55	0.03	12.4		53	0.03	11.4	
	Catalonia Direction I				**Direction II**			
	df	MS	% var.	*p*	df	MS	% var.	*p*
distance	6	0.65	32.8	***	5	0.63	25.3	***
site (distance)	12	0.24	34.1	***	12	0.24	37.0	***
Error	45	0.05	33.1		43	0.05	37.7	

Regression equations fitted to the $\delta^{15}N$ data normalized to the control mean (i.e. relative net increments) and the calculated maximum distances of dispersal, are shown in Table 4 and represented in Figure 4. The maximum distance at which the nitrogen is dispersed from the fish farm was different both between study sites and directions (DI and DII). In the Canary Islands fish farm, this distance was 4-fold higher in DI (570 m) than in DII (138 m). Nonetheless in DII, $\delta^{15}N$ signatures were 16% higher than reference values indicating that the 1000 m distance was not an appropriate control in this case and hence the maximum distance estimated for DII is likely an underestimation of the real extent. In the Murcia study case, the calculated maximum distances were the highest of the three study sites. In the first experiment (A), the maximum distance was 3-fold higher in DI (2006 m) than in DII (675 m) and showed an opposite spatial pattern in the second experiment (B) with dispersal distances 1.8-fold higher in DII (1458 m) than in DI (800 m). The maximum distance obtained for the Catalonia fish farm was the shortest of the three fish farms studied, with 76 m in DI and 106 m in DII.

Table 4 Summary of statistic results of different regression models between $\delta^{15}N$ values of macroalgae and distances from fish farm in different study sites and for each study direction (DI and DII). Distances below y= 0.2 correspond to the maximum area of influence of fish farms wastes. For Murcia the results of two identical but separated in the time experiments are presented (Murcia A and Murcia B). * p< 0.05; ** p < 0.01; *** p<0.001; ns= not significant.

Fish farm	Var.	Direction	Regression equation y = a*exp (-b*x)	df	R^2_{adj}	F_{reg}	*p-value*	Maximum distance (m)
Canary Islands	$\delta^{15}N$	I	y = 0.93*exp (-0.0030*x)	6	0.90	55.2	***	570 ± 128
		II	y = 1.08*exp (-0.0122*x)	6	0.95	117.8	***	138 ± 20
Murcia A	$\delta^{15}N$	I	y = 0.81*exp (-0.0007*x)	6	0.82	28.4	**	2006 ± 450
		II	y = 0.82*exp (-0.0021*x)	6	0.82	27.7	**	675 ± 221
Murcia B	$\delta^{15}N$	I	y = 0.84*exp (-0.0018*x)	6	0.89	47.2	**	800 ± 173
		II	y = 0.86*exp (-0.0010*x)	6	0.86	36.5	**	1458 ± 364
Catalonia	$\delta^{15}N$	I	y = 1.05*exp (-0.0217*x)	6	0.70	12.7	*	76 ± 51
		II	y = 1.03*exp (-0.0156*x)	6	0.94	67.9	**	106 ± 18

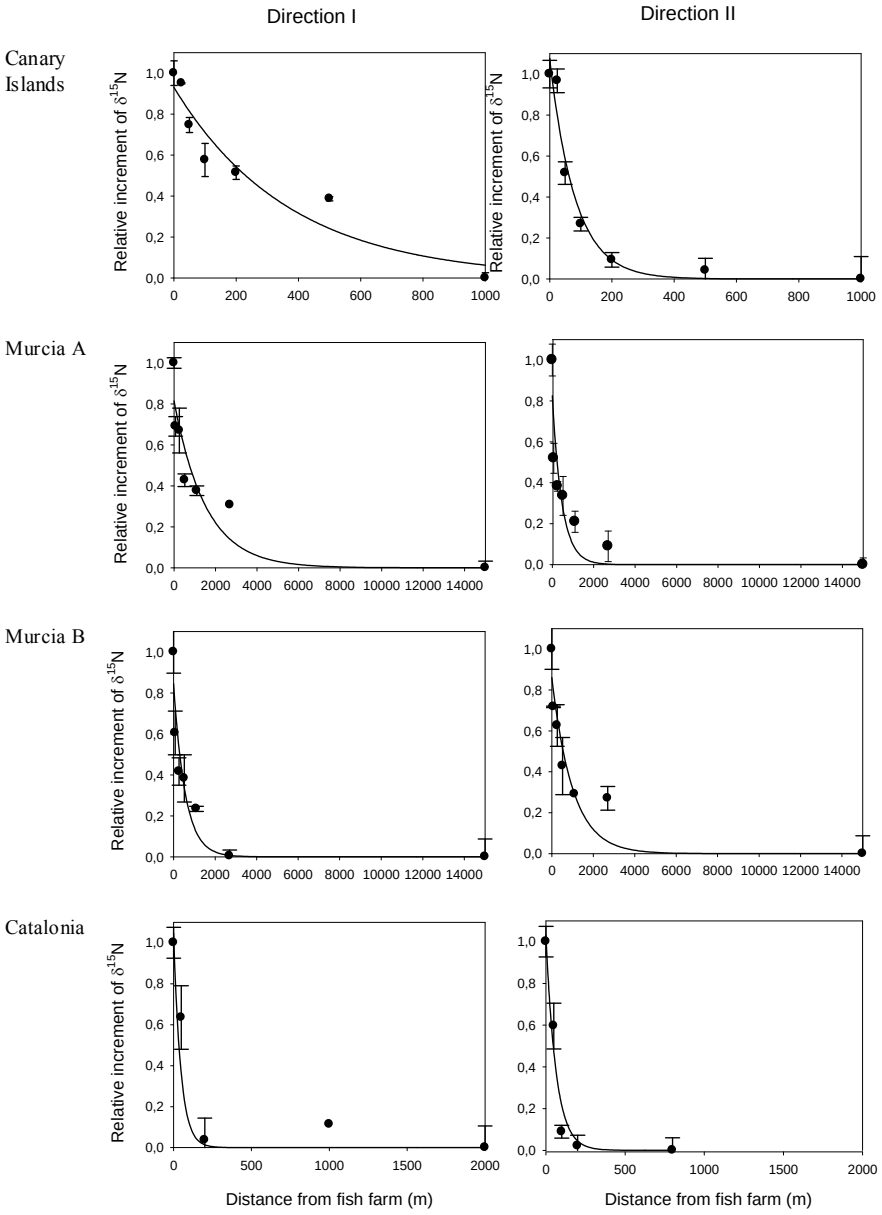


Figure 4: Graphic relationship between nitrogen isotopic ratio ($\delta^{15}N$) in macroalgae and distances from fish farm in the Canary Islands (*Stypopodium zonale)*, Murcia (*Dyctiopteris polypodioides)* and Catalonia (*Cystoseira mediterranea)*. The results of macroalgae for two directions (DI and DII) are shown. For Murcia the results of two identical but separated in the time experiments are presented (Murcia A and Murcia B). Vertical bars correspond to the standards error of the mean.

Total N content (% N)

Total nitrogen content (% N) in the macroalgal bioassays deployed at control sites showed similar values to reference samples (p-value>0.05) in all study cases except in Catalonia where control values of % N were lower than the reference values (p-value<0.05; Table 2). The analysis of % N in macroalgal bioassays deployed at different distances from fish farms showed significant differences between incubation points along the two transects directions of each study site (Fig. 5 and Table 5). At the Canary Islands fish farm, mean % N values of *Stypopodium zonale* increased from 1.4 ± 0.05 % at control sites to 2.5 ± 0.1 % at sites closest to the farm in the direction DI and from 1.5 ± 0.03 % to 2.1 ± 0.1 % in the direction DII. Mean values were significantly higher than control means down to the 25 m distance for DI and up to 500 m distance for DII (SNK, p=0.05; Fig. 5). The distance from fish farm accounted for a 48.7 to 63.3 % of the total variation, but the relative contribution of the variability between bioassays (i.e. error term) was equally important (36.7-51.0 %) (Table 5). In the Murcia study case, mean % N values of *Dyctiopteris polypodioides* obtained in the first experiment (Murcia A) increased from 1.9 ± 0.06 % at control sites to 2.9 ± 0.1 % near the farm in the directions DI and DII. For both directions, mean values were maintained higher than control means up to the 2,700 m-distance (SNK, p=0.05; Fig. 5). In the second experiment (Murcia B) mean % N values increased from 1.9 ± 0.06 % at control sites to 2.7 ± 0.08 % near the farm in the direction DI and from 1.9 ± 0.06 to 2.7 ± 0.05 % in the direction DII. In this experiment, mean values were significantly and clearly higher than control means up to 1,100 m in DI and 2,700 m in DII (SNK, p=0.05; Fig. 5). In this study case, variability of % N values in macroalgal bioassays were mainly accounted by the distance from fish farm (58.8-79.9 %; Table 5). In the Catalonia study case, % N values of *Cystoseira mediterranea* increased from 1.1 ± 0.03 % at control sites to 1.3 ± 0.02 % near the fish farm in the direction DI and from 1.2 ± 0.04 to 1.4 ± 0.02 % in the direction DII. Mean values were significantly and clearly higher than control means down to the 25 m-distance for both directions. In this study case, variability of % N values in macroalgal bioassays were mainly accounted by the variability between bioassays (i.e. error term) (55.1-59.7 %; Table 5).

Table 5 Summary of results of a nested ANOVA and estimated variance component (% var.) on total N content (% N) in macroalgae tissues incubated in three different study sites at different distances from fish farm. Each distance was replicated three times along two different directions (DI and DII). For Murcia the results of two identical but separated in the time experiments are presented (Murcia A and Murcia B). * p< 0.05; ** p < 0.01; *** p<0.001; ns= not significant.

	Canary Islands							
	Direction I				Direction II			
	df	MS	% var.	*p*	df	MS	% var.	*p*
distance	6	1.37	63.3	***	6	0.61	48.7	***
site (distance)	14	0.05	0.0	ns	14	0.05	0.3	ns
Error	63	0.06	36.7		62	0.05	51.0	
	Murcia A							
	Direction I				Direction II			
	df	MS	% var.	*p*	df	MS	% var.	*p*
distance	6	1.68	65.3	***	6	1.50	58.8	***
site (distance)	13	0.09	2.7	ns	14	0.11	4.7	ns
Error	60	0.06	32.0		59	0.07	36.5	
	Murcia B							
	Direction I				Direction II			
	df	MS	% var.	*p*	df	MS	% var.	*p*
distance	6	1.62	67.2	***	6	1.87	79.9	***
site (distance)	13	0.08	3.2	ns	11	0.04	1.1	ns
Error	58	0.06	29.6		52	0.04	19.0	
	Catalonia							
	Direction I				Direction II			
	df	MS	% var.	*p*	df	MS	% var.	*p*
distance	6	0.03	14.7	***	5	0.05	23.7	***
site (distance)	12	0.02	25.6	**	12	0.02	21.2	ns
Error	55	0.01	59.7		45	0.01	55.1	

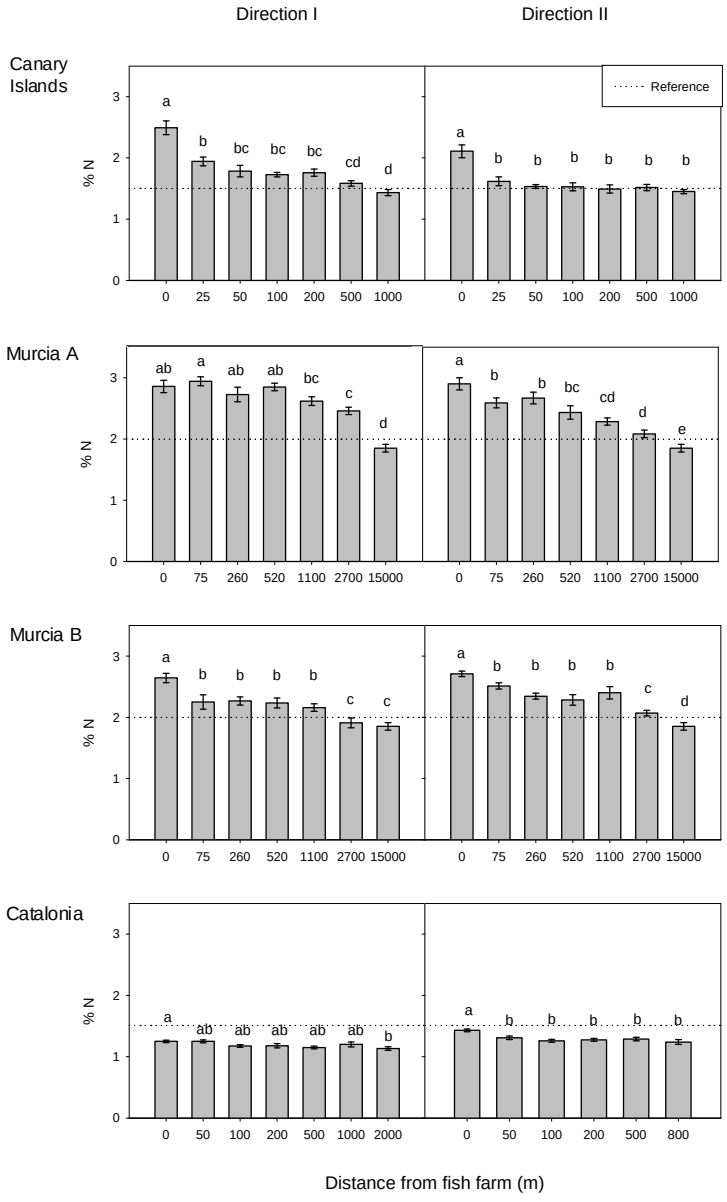


Figure 5: Total nitrogen content (% N) in macroalgae incubated at different distances from fish farm in the Canary Islands (*Stypopodium zonale)*, Murcia (*Dyctiopteris polypodioides)* and Catalonia (*Cystoseira mediterranea*). For each species of macroalgae the results for two directions (DI and DII) are shown. For Murcia the results of two identical but separated in the time experiments are presented (Murcia A and Murcia B). Vertical bars correspond to the standards error of the mean.

Discussion

Our results show that the analysis of nitrogen stable isotope ratio ($\delta^{15}N$) in macroalgae bioassays is a reliable tool for quantitative characterizations of the spatial patterns of farm wastes dispersal in off-shore environments. These findings agree with results from similar incubation experiments carried out by Lin and Fong (2008) and Costanzo et al. (2001), in relation to the spread of shrimp farm and urban wastes, respectively, in coastal environments. The increment of $\delta^{15}N$ in macrophyte tissues incubated close to the fish cages (in relation to control distances and reference values) reflect the enrichment in this isotope typically measured in aquaculture wastes (7-11 ‰ Sarà et al., 2004; Lojen et al., 2005; Vizzini and Mazzola, 2006) and in any other dissolved and particulate sources of anthropogenic origin (7-13.5 ‰ e.g. Costanzo et al., 2001; Cole et al., 2004; Deutsch and Voss, 2006). Similarly, decreasing trends of $\delta^{15}N$ values across distances from fish cages reported in this study can be explained by the mixing of farm wastes with oceanic sources, which typically show lower $\delta^{15}N$ signatures (3-4 ‰ e.g. Lojen et al., 2005). The ability of macrophytes to directly reflect the availability and isotopic composition of N sources is due to its capacity to uptake and store N excess in their tissues and the small or null fractionation during N uptake across a wide range of nutrient concentrations (Cohen and Fong, 2005; Lin and Fong, 2008). Therefore, spatial patterns of $\delta^{15}N$ described in this study using macroalgae bioassays clearly represent the dilution of farm wastes as they are dispersed away from fish farms by local hydrodynamic forces.

In the Murcia study cases (A and B), there was a good agreement between changes in N and $\delta^{15}N$ observed in tissues of *D. polypodioides* across the incubation distances, likely because N availability is enough high to merit storage of N excess in their tissues. However, this was not the case of the Canary Islands experiment, and especially for the bioassays deployed along the DII direction (Figs. 3 and 5). In this case, total N content of *S. zonale* decreased to control and reference values immediately after the 0 meters distance (i.e. between 25 and 1000 m), but high

$\delta^{15}N$ values were observed even at the largest distance (1000 m, 16.1% higher than mean reference values). In absence of other anthropogenic N sources in the area, this result suggest that the influence of farm wastes extended beyond the 1 km distance and hence it can not be considered as an appropriate control in this case (see results section also). This discrepancy between total N content and $\delta^{15}N$ values was also reported by Lin and Fong (2008) using the tropical macroalga *Acanthophora spicifera* in a similar experiment aimed to test the effectiveness of macroalgal bioindicators in assessing nutrient enrichment caused by shrimp farm wastes. These authors explained such discrepancy by the fact that excess nitrogen was being used primarily for growth and no long term storage of N took place, as has been experimentally demonstrated for other macroalgae species (e.g. Fong et al. 2004). Since we did not measure algal growth rates, we can not confirm such hypothesis in our case, but these results support the general contention that N isotopes are more sensitive to dissolved nutrients, as they are able to detect the influence of anthropogenic N sources at larger distances and lower concentrations (Sarà et al., 2004; Jones et al., 2001; Lin and Fong, 2008; Ruiz et al., in press). Comparison between total N content and $\delta^{15}N$ values of *Cystoseira mediterranea* in the Catalonia study case also support such contention.

Differences in spatial patterns of N content and $\delta^{15}N$ in macrophytes could be accounted by species-specific characteristics of their nutrient physiology and growth (e.g. Deutsch and Voss 2006, Gartner et al., 2002), but at least a part of our results suggest that such differences respond to farming and environmental features more than to taxonomic factors. Thus, differences in maximum distances of waste dispersal (i.e. calculated from regression equations fitted to $\delta^{15}N$ data) for Murcia and Canary Islands fish farms match with their contrasting differences in fish production, food supply and cultured fish species of both facilities. Mean maximum distances obtained in the Murcia study cases were in the range 600-2000 m, which is up to one order of magnitude higher than those obtained in the Canary Island study case (100-570 m). Accordingly, annual fish production in the Murcia farm complex was 16-fold higher than in the Canary Island farm. Furthermore, blue fin tune cultured in the Murcia farm are

known to have lower feed conversion efficiencies (20/30:1; Vita et al., 2004; Aguado-Giménez and García-García, 2005) and higher production of dissolved N wastes (mg N Kg $fish^{-1}$ day^{-1}; Aguado-Giménez et al., 2006) than in conventional species cultured in the Canary Island farm. These results agreed with the positive correlation founded between $\delta^{15}N$ values in aquatic macrophytes and total N loads entered into aquatic ecosystems (e.g. Cole et al., 2005). However, results obtained for the Catalonia farm does not fit well to this relationship. In this case the maximum dispersal distance was of only 106 m but fish production was more than twice that that sustained by the Canary Island farm. In effect, *C. mediterranea* bioassays showed the lowest capacity to reflect both N excess and isotopic N signatures of farm wastes: increment of mean values of both variables at the 0 m-distance (9-15%) was considerably lower that that observed in the Canary Island farm at the same position (26-74%). In absence of additional information, there is not a clear explanation for this reduced sensitivity to nutrient availability, but some ideas can be addressed. In the case of $\delta^{15}N$, high initial values of *C. mediterranea* (5.9 ‰) prevent further increase in incubated tissues since these values are very close to the N signature of farm wastes. A similar situation was described for other macroalga species unable to reflect demonstrated gradients of nutrient availability along a estuary (i.e. *Fucus vesiculosus*, Deutsch and Voss, 2006); in the other two study cases, the higher responses observed are consistent with the lower initial values of the alga species used, which are closest to isotopic N signals reported for natural water column sources (2-4 ‰; Costanzo et al., 2001; Cole et al., 2004). The initial $\delta^{15}N$ values of *C. mediterranea* was much higher that those measured in other Mediterranean *Cystoseira* spp growing in pristine environments (2.26 ± 0.1 ‰, Pinnegar and Polunim, 2000) and close to the $\delta^{15}N$ values of anthropogenic DIN sources (5.2-7.8 ‰) (Umezaba et al., 2002; Deutsch and Voss, 2006). Therefore, is possible that large-scale gradients of anthropogenic inputs from the neighbour Ebro River are masking the effect of spatial nutrient gradients of farm wastes. The presence of anthropogenic nutrient sources, different to that we wish to trace, is in fact an important confounding factor in this kind of applications of the isotope techniques (Fourqurean et al., 1997; Lepoint et al., 2004). Species-specific characteristics (e.g. nutrient uptake and growth rates) are also responsible for the

differential sensitivity of incubated macroalgae species to altered N availability and isotopic composition (e.g. Gartner et al., 2002, Deutsch and Voss, 2006). *C. mediterranea* belongs to a functional form group with lower growth and uptake rates than foliose forms similar to the other species tested (Wallentinus, 1984), but more precise data would be required to consider this possibility.

The analysis performed in this study also indicate a clear spatial anisotropy of waste dispersal, as maximum distances of the extent of waste dispersal obtained with $\delta^{15}N$ data were always higher in one direction than in the other. This effect has been typically described in simulations of farm waste dispersal obtained from computing particle tracking models using current meter data (e.g. DEPOMOD model, Cromey et al., 2002), and merely reflect the direction and speed of the dominant current. Relationships between current speed and direction and the spatial extent of farm wastes have been demonstrated by Sarà et al. (2006). We have not quantitative data of local currents during the experimental periods, but the largest distance obtained in each case coincided with the direction of dominant winds and surface currents observed in the field during that period. In the Murcia study case, the change in the main dispersion directions between experiments A (DI) and B (DII) was consistent with changes in wind and surface current directions due to changing weather conditions. Previous works performed in the Murcia study site (Ruiz et al., in press) showed that elevated $\delta^{15}N$ signatures measured in the epiphytic community of a seagrass (*Posidonia oceanica*) meadow located 2.8 km SW the fish farm was mainly caused by the dispersal of farm wasted in this direction. In the Murcia experiment A, the largest dispersal distance was observed just in that direction (i.e. DI, SW) and was estimated in ca. 2 km. Therefore, at least in this case, there is a high correspondence between the spatial extent of farm wastes estimated from our bioassay experiment and that measured in nearby benthic communities.

In conclusion, results obtained in this study demonstrate that the macroalgal bioassay used can provide reliable and useful information to determine the spatial extent of off-shore

farm wastes at a large range of spatial scales. Since this information is crucial to evaluate environmental risk on sensitive habitats (and hence for management of the off-shore aquaculture industry in many coastal zones), its advantages and effectiveness must be considered as a real alternative or complement to the traditional methods based on physico-chemical measurements of water quality. Furthermore, the bioassay method has been shown to be sensitive to reflect changes in husbandry parameters (e.g. total waste production) and local hydrodynamic conditions (e.g. speed and direction of currents) and hence can be a very useful tool to calibrate numeric models traditionally used to simulate waste dispersions. Nonetheless, some limitations and constrains of the method are highlighted from this study. Thus, for example, care must be taken in cases where other N sources can confound the bioassays responses to the fish farm wastes. Also, previous knowledge of the nutrient physiology of macroalga species would be required. Pilot studies should be conducted to solve these questions prior to the systematic application of the bioassay methods in the assessment of aquaculture impacts.

Chapter 5

Experimental evidence supports the use of $\delta^{15}N$ in macroalgal tissues as an indicator of available N sources

Evidencia experimental que apoya el uso de δ^{15}N en tejidos de macroalgas como indicador de fuentes disponibles de N

Resumen

El uso de la composición isotópica del nitrógeno (δ^{15}N) en tejidos de macroalgas para determinar fuentes de nitrógeno (N) se basa principalmente en el siguiente supuesto: el δ^{15}N de los productores primarios refleja las fuentes de nitrógeno de manera predecible. Para comprobar esta hipótesis, se llevaron a cabo varios experimentos de laboratorio con la macroalga *Cystoseira mediterranea* (Sauvageau). En el primero, se modificó el δ^{15}N del amonio de agua de mar manteniendo una concentración de NH_4^+ constante para determinar si las macroalgas reflejan la señal isotópica del medio. El δ^{15}N de los tejidos incrementó con el δ^{15}N suministrado; por lo tanto, no se produjo una selección de ^{14}N frente al ^{15}N (fraccionamiento). En el segundo experimento, mantuvimos el δ^{15}N constante y variamos la concentración de NH_4^+ para determinar si se producía fraccionamiento dependiente de la concentración. No hubo relación entre la concentración de amonio del agua de mar y el δ^{15}N de tejidos por lo que no se encontró fraccionamiento dependiente de la concentración. Finalmente, se incubaron macroalgas expuestas a piensos de pescado para corroborar que las macroalgas pueden reflejar la señal isotópica de esta fuente de N. Este último experimento se realizó bajo diferentes condiciones de luz para evaluar el efecto de la variabilidad de la intensidad lumínica en el fraccionamiento de macroalgas. Nuestros resultados mostraron que *C. mediterranea* refleja mejor la señal isotópica de los vertidos de las granjas marinas en condiciones de luz que en condiciones de sombra. Este estudio demuestra que la acumulación de ^{15}N en tejidos de macroalgas es predecible en un rango de δ^{15}N y de concentraciones de N y que algunos factores ambientales, tales como la intensidad de luz, han de tenerse en cuenta a la hora de usar *C. mediterranea* para evaluar la disponibilidad de fuentes de N en estudios ecológicos.

Abstract

The use of stable nitrogen isotope ratios ($\delta^{15}N$) in macroalgal tissues for determining nitrogen (N) sources is based mainly on the assumption that the $\delta^{15}N$ of primary producers reflects N sources in a predictable manner. To test this assumption, we conducted several laboratory experiments with the macroalgae *Cystoseira mediterranea* (Sauvageau). First, we varied $\delta^{15}N$ in seawater ammonium but with constant concentration of NH_4^+ to determine whether macroalgae reflects the isotopic ratio of the medium. Tissue $\delta^{15}N$ increased with $\delta^{15}N$ supplied; therefore no selection for ^{14}N over ^{15}N occurred (fractionation). Second, we held $\delta^{15}N$ constant and varied concentrations of NH_4^+ to determine whether fractionation was concentration dependent. We found that there was no relationship between ammonium concentration in seawater and tissue $\delta^{15}N$, so no fractionation concentration dependent was found. Finally, we incubated macroalgae exposed to fish food to corroborate than macroalgae can reflect the isotopic signal of this N source. We perform this last experiment under varying light conditions to evaluate the effect of light variability on macroalgae fractionation. Our results showed that *C. mediterranea* reflect better the isotopic signal of fish farm wastes when it is exposed to high light conditions than to low light conditions. This study demonstrated that accumulation of ^{15}N in macroalgal tissue was predictable over a range of water $\delta^{15}N$ and N concentrations and that some environmental factors, as light intensity, must be taken into account for to use *C. mediterranea* to assess the availability of N sources in ecological studies.

Keywords: Nitrogen Stable isotopes ($\delta^{15}N$), Macroalgae, Light, Indicator.

Introduction

Increasing human populations in coastal areas worldwide have caused increases in environmental and ecological change (Halpern et al., 2008). One of the principal impacts of human population growth is the increased production of nitrogen and phosphorus. Changes in the levels of nutrient availability have been shown to cause changes in the physical and chemical properties of the marine environment and changes in abundances of species in coastal ecosystems, including seagrass disappearance worldwide (e.g. Short and Wyllie-Echeverria, 1996; Bokn et al., 2002; Tewfik et al., 2005; Burkholder et al., 2007). However, despite eutrophication has been invoked as a major cause of ecosystems degradation, the movement of nutrients through ecosystems continuous being difficult to measure directly.

Stable nitrogen isotope ratios (δ^{15}N) are increasingly using to determine nitrogen sources to ecosystems in ecological studies (Peterson and Fry, 1987; Aravena et al., 1993; McKinney et al., 2001; Lepoint et al., 2004; Fry, 2006). Many of these studies use natural abundance of δ^{15}N in marine plants and algae as a tool to identified sources of nitrogen enrichment across a wide range of ecological systems (Erskine et al., 1998; McClelland and Valiela, 1998; Cole et al., 2004; Kaushal et al. 2006). These organisms reflect not only conditions at the time of sampling but also conditions to which the community was previously exposed showing a time-integrated estimate of exposure to natural and anthropogenic nitrogen sources. The analysis of δ^{15}N in macroalgal tissues has been used with success to detect the spatial extent of land derived sewage (Costanzo et al., 2001; Gartner et al. 2002; Savage and Elmgren 2004) as well as indicator of fish-farm waste dispersal (Jones et al., 2001; Vizzini and Mazzola, 2004; Pérez et al., 2008; García-Sanz et al., in press). However, the use of marine algae in ecological studies based in the use of stable isotopes relies on an understanding of how isotope signatures of algal tissue reflect inorganic source nitrogen isotope signatures, and how these varies under varying physical conditions.

Nitrogen stable isotopes are viewed as useful tracers of enrichment because different sources of nitrogen often have characteristic and widely divergent signatures (Heaton, 1986). The underlying assumptions of the use of this technique are that the $\delta^{15}N$ of sources are known and that primary producers take up ^{15}N from the environment in proportion to availability in a predictable manner (Cifuentes et al., 1988). However, knowing the $\delta^{15}N$ of sources may not be enough to establish where the N in primary producers was originated, because different N isotopes may not be taken up equally. Specifically, this approach will require that no discrimination between ^{14}N and ^{15}N occurs during uptake and posterior assimilation of nitrogen by the primary producer.

For marine plants and algae, the process of discrimination between ^{14}N and ^{15}N that results in a difference between the $\delta^{15}N$ signature of inorganic nitrogen in seawater, and the $\delta^{15}N$ signature of the plant (i.e. 'fractionation') may occur at several stages. Discrimination during incorporation of DIN from seawater into algal tissues may occur during active uptake of DIN over the plasma membrane, and/or during reduction of NO_3^- to NO_2^- and subsequently to NH_4^+ (catalysed by nitrate reductase and nitrite reductase respectively), and finally during synthesis of amino acids and subsequent compounds (Needoba et al., 2004). If discrimination takes place within the algal cell to result in a fractionation effect between seawater DIN and algal tissue, heavier isotopes (which form slightly stronger chemical bonds), will be less represented in the product pools of these processes. Therefore, some of the isotopically heavier reactant pools need to be effused from the plant.

Fractionation has been found to be concentration dependent with higher N availability across some primary producer groups (higher plant, bacteria, microalgae), resulting in a greater uptake of ^{14}N and therefore lower $\delta^{15}N$ in tissue (Wada and Hattori, 1978; Mariotti et al., 1982; Hoch et al., 1992; Waser et al., 1998a; McKee et al., 2002). For example, Pennock et al. (1996) examined diatom N fractionation under enriched conditions and found selection for ^{14}N at all but the lowest concentrations (<20μM). Others works have found that light availability

(Heikoop et al., 1998; Needoba and Harrison, 2004) and N source (e.g. ammonium, nitrate) (Waser et al., 1998b) may also have some effect on fractionation during nitrogen incorporation in photoautotroph. However, to our knowledge, it is very little information available evaluating or even describing predictability of ^{15}N behaviour in macroalgae under varying levels of nutrient availability or environmental conditions (but see Cohen and Fong, 2005; Cornelisen et al., 2007). The few studies performed have used opportunistic green macroalgae (*E. intestinalis* and *U. Pertusa*). These studies provided evidences demonstrating that the species studied do not exhibit N isotope fractionation since variations in δ^{15}N ratios were consistent with expected changes in the available source and signature of dissolved inorganic nitrogen assimilated by the algae.

In this study we use controlled laboratory experiments to investigate if N fractionation occurred in the macroalga *Cystoseira mediterranea* (Sauvageau) under variable nitrogen and light environments. Experimental treatments are guided by environmental conditions found at a Spanish coastal site impacted by a fish farm discharge (García-Sanz et al., in press). The concrete goals of this study were to investigate if N fractionation occurred in this macroalga under varying nitrogen availability (experiment 1 and 2) and corroborate that *C. mediterranea* reflects changes in its isotopic signal when it is exposed to fish farm food to be used as indicator of fish farm wastes as well as investigate if N fractionation occurred in this macroalga under varying light conditions (experiment 3). To accomplish these goals, we measured algal responses including total N and δ^{15}N content.

Materials and Methods

Sample collection. Pieces of the macroalgae *Cystoseira mediterranea* (Sauvageau) were collected from unpolluted rocky intertidal sites of the coast of L´Ametlla de Mar (Catalonia,

Spain). Species of the genus Cystoseira (Fucales, Cystoseiraceae) dominate Mediterranean upper sublittoral communities (Feldmann, 1937). This highly structured and productive community is observed in hydrodynamic environments and non-polluted waters (Ballesteros, 1988) and is particularly sensitive to any natural or anthropogenic stress (Bellan-Santini, 1968; Ballesteros et al., 1984; Hoffmann et al., 1988; Soltan et al., 2001). The collected algae were preincubated during one night in coolers tanks with ample aeration and low light conditions to avoid further physiological stress by light and temperature, and in low-nutrient seawater (Kamer and Fong, 2001; Kamer et al., 2001). Six sub-samples were separated from the bulk of collected plant material to obtain natural range values of the variables analyzed for our experimental treatments ($\delta^{15}N$ and total % N). All the experiments were performed between July and September 2007 at the University of Barcelona, Spain.

Experiment 1. We conducted an experiment to determine if isotopic fractionation occurs in *Cystoseira mediterranea* during nitrogen uptake and assimilation. We varied $\delta^{15}N$ (control, 5, 10 and 20 ‰) in NH_4^+ but maintaining ammonium concentration constant and high enough to be non limiting. If there is not fractionation, we predict that the proportion of the two isotopes in the tissues will be the same as in the water ammonium.

Macroalgae pieces (between 4.5 and 5 g of plant biomass**)** were incubated inside glass jars (1.5 L) under controlled light and temperature conditions. Saturating irradiance was provided by fluorescent lamps (300-400 μM photons m^{-2} s^{-1}) and algae were exposed to light cycles of 12 h. Temperature varied between 23 (night) and 29ºC (day) and adequate aeration and mixing was ensured by diffusers and air pumps. Treatment water was created by adding known amounts of isotopically heavy and light $ClNH_4$ (Isotec, Inc., Miamisburg, OH, USA) to low-nutrient seawater. Specifically, we prepared three classes of treatment waters adding 20.0414, 20.1395 and 20.3411 μg/l of $ClNH_4^{15}$ and 5.32932, 5.32923 and 5.32903 mg/l of $ClNH_4^{14}$ respectively to obtain solutions of 100μM with $\delta^{15}N$ of 5, 10 and 20 ‰ each one. Initial isotopic ratio did not affect the final $\delta^{15}N$ of the experiment treatments because concentrations of added nutrients

were several orders of magnitude greater than the background concentrations. In each experimental unit deployed (n=3) we added 1 l of treatment water. Algae were incubated during three days. Each day, the water of each jar was changed to maintain ammonium concentration around 100μM NH_4^+. This N concentration was high enough to avoid nutrient depletion throughout the 24-h duration and was chosen according the results of a previous experiment (non published results) where water samples from each experimental unit were analyzed for ammonium concentration to ensure that depletion of N was not occurring. After exposure to treatments, algal samples were analyzed for total nitrogen content (% N) and δ^{15}N.

Experiment 2. To determine if there is fractionation dependent on ammonium concentration, one-factor experiment was conducted varying NH_4^+ concentration but holding δ^{15}N constant. If selection for ^{14}N occurs, one would predict that as concentration decreases, ^{14}N availability decreases, ^{15}N will then be used, so tissue δ^{15}N will increase. Thus, fractionation would result in a negative relationship between ammonium concentration and δ^{15}N. We exposed algal tissue to 70, 100, 130, 160 and 200 μM solutions of NH_4Cl during 24-h (n=2). Experimental treatment water was created by mixing known amounts of heavy and light NH_4Cl in low nutrient seawater to get a 500- μM N solution with a δ^{15}N approximately 5 ‰. We diluted the stock water with low-nutrient seawater to achieve a range of concentration. Experimental setup and culture conditions were as described for the first experiment.

Experiment 3. A two-way experiment was performed to evaluate if δ^{15}N values of macroalgae tissues exposed to fish farm food reflect the δ^{15}N of the source. We perform this experiment varying light conditions to determine whether fractionation was light dependent. Macroalgae were incubated during 24-h in treatment water, created by mixing a known amount of fish food (5 g) in low-nutrient seawater. The two experimental light conditions were High Light (300-400 μM photons m^{-2} s^{-1}) and Low Light (50-100 μM photons m^{-2} s^{-1}). Collection, experimental setup and culture conditions were as described earlier. δ^{15}N of particulate fish farm food were also measured.

Laboratory analyses. Ammonium concentration in the water samples of the experiment 1 was determined by the phenol-hypochlorite method (Solorzano, 1969). For the rest of analysis, algal tissue was removed from the experimental units, spun for 1 min in a lettuce spinner to remove excess water, wet weighed, briefly rinsed with deionized water to remove external salts, and dried at 60ºC to constant weight. Dried samples were homogenized by grinding with a mortar and pestle, and preserved in a desiccator at room temperature. A subsample was analyzed for tissues N content (% N total) and $\delta^{15}N$. Total N and $\delta^{15}N$ content were determined using a continuous flow IRMS (ThermoFinnigan FlashEA1112 and a mass spectometer DELTAplus with an interface of continuous flow ConFlo II of Finnigan MAT) for high precision analysis of combusted solid samples at natural abundance and for analysis of trace gases. The ^{15}N data ($\delta^{15}N$) were presented as the per mil (‰) deviation of that sample from the isotopic composition of a reference compound:

$$\delta^{15}N\ (‰) = (R_{sample} / R_{standard}) - 1) \times 10^3$$

where, R is the isotope ratio (^{15}N: ^{14}N) and the reference compound is atmospheric N_2 with a ratio of (0.0036765). Therefore, the $\delta^{15}N$ of air ($1000 \cdot [(0.0036765/0.0036765)-1]$) is considered to be 0 ‰ for $\delta^{15}N$ measurements (Hoch et al., 1992; Waser et al., 1998 a,b).

Statistical analysis. Data were tested to ensure they met the assumption of parametric statistics by examining frequency histograms of the raw data as well as the distribution of residuals. No transformations were necessary. Differences between treatments in the different experiment were analyzed by one or two-factor ANOVA d a post-hoc Tukey honest significant difference (HSD) tests were used to examine the differences found significant by ANOVA. Statgraphics Plus 5.1 data analysis software package was used for the statistical analysis.

Results

Experiment 1. Mean values of nitrogen stable isotope ratios (δ^{15}N) and total N content (% N) in *Cystoseira mediterranea* tissue collected from unpolluted sites were 6.6 ± 0.3 and 1.4 ± 0.04, respectively (n=6). Tissue δ^{15}N values obtained in this experiment were several orders of magnitude higher than those expected (5, 10 and 20 ‰). This was probably due to the difficulty in the weight of the extremely low quantities of $ClNH_4^{15}$ added to the experimental water. But although the values were higher than the expected, the increments were maintaining following the δ^{15}N supplied (Fig. 1 A, Table 1). These results follow our prediction that tissue δ^{15}N show a significant increase with increasing water δ^{15}N, so fractionation do not occurs. Total N content was similar in the three treatments (Fig. 1 B, Table 1).

Table 1: Results of one-factor ANOVA testing the influence of isotope ratios (experiment 1) and nutrient concentration (experiment 2) on δ^{15}N and % N in macroalgal tissues.

	Variable	SS	df	MS	F	*p*
Experiment 1	*$\delta^{15}N$*					
	Between	1,683,090	2	841,546.0	63.23	0.0000
	Within	292,814	22	13,309.7		
	Total	1,975,910	24			
	%N					
	Between	0.2506	2	0.1253	2.24	0.1302
	Within	1.2306	22	0.0559		
	Total	1.4812	24			
Experiment 2	*$\delta^{15}N$*					
	Between	14,206.1	4	3,551.53	3.24	0.1408
	Within	4387.5	4	1,096.87		
	Total	18,593.6	8			
	%N					
	Between	0.4765	4	0.1191	31.85	0.0009
	Within	0.0187	5	0.0037		
	Total	0.4952	9			

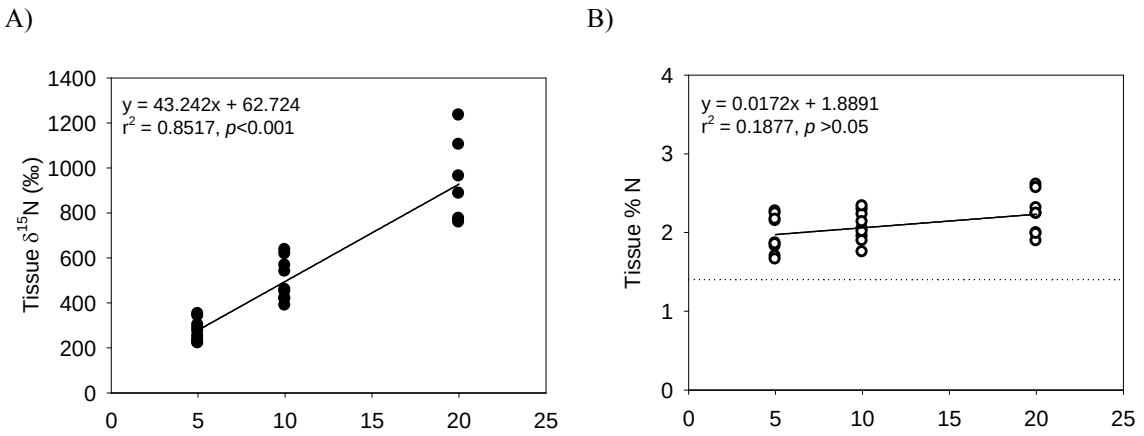


Figure 1: Values of A) nitrogen stable isotope ratios ($\delta^{15}N$) and B) total N content (% N) in *Cystoseira mediterranea* tissue exposed to different treatments (5, 10 and 20 ‰). Dotted lines represent initial $\delta^{15}N$ and % N content.

Experiment 2. There was no relationship between NH_4^+ concentrations and tissue $\delta^{15}N$ (Fig. 2 A, Table 1) suggesting that fractionation did not occurs with increasing seawater N availability. If fractionation were occurring, we expected that tissue $\delta^{15}N$ would increase with decreasing N availability and it did not. On the other hand, we found an increase in total N content in macroalgae tissue following an increase in NH_4^+ availability (Fig. 2 B, Table 1).

Experiment 3. Mean values of nitrogen stable isotope ratios ($\delta^{15}N$) and total N content in the fish food used in this experiment were 9.7 ± 0.3 and 7.5 ± 0.2, respectively (n=6). Tissue $\delta^{15}N$ was significantly higher in macroalgae exposed to fish food than in control algae (Fig. 3 A, Table 2). $\delta^{15}N$ ratios in *Cystoseira mediterranea* tissues exposed to fish food were close fish food ratios and they were significantly higher in High Light treatment than in Low Light treatment indicating the existence of light dependent fractionation. Total N content was similar in the two treatments with no differences between total % N of control and fish food treatment and between High light and Low Light conditions (Fig. 3 B, Table 2).

Table 2: Results of two-way ANOVA examining the influence of nutrient source and light treatment (experiment 3) on δ^{15}N and % N in macroalgal tissues.

Variable	Source of variation	SS	df	MS	F	*p*
δ^{15}N	Nutrient	20.65	1	20.65	15.21	0.0080
	Light	5.16	1	5.16	3.80	0.0991
	Nutrient*Light	8.36	1	8.36	6.16	0.0477
	Residuals	8.15	6	1.36		
% N	Nutrient	0.12	1	0.12	2.12	0.1837
	Light	0.12	1	0.12	2.12	0.1837
	Nutrient*Light	0.00	1	0.00	0.06	0.8145
	Residuals	0.45	8	0.06		

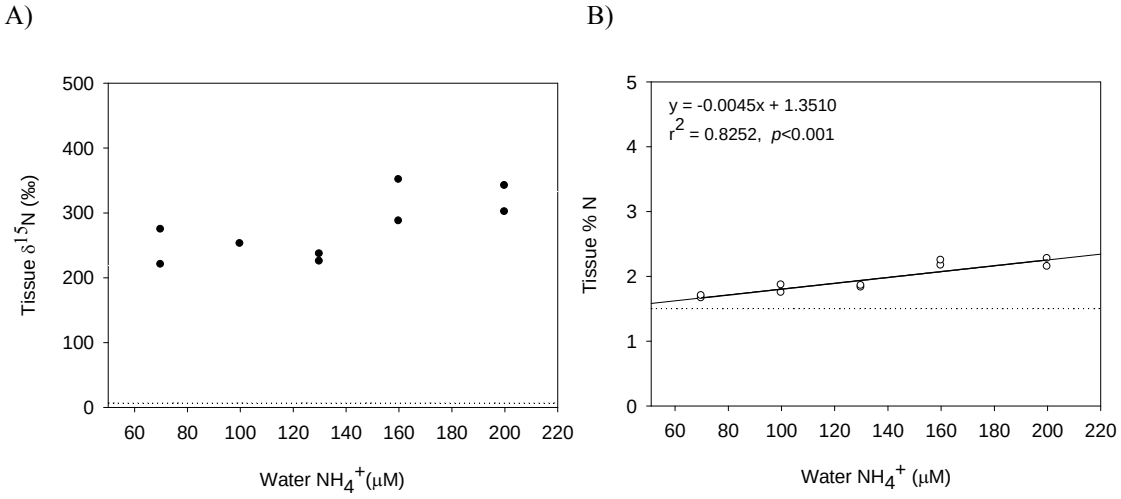


Figure 2: Values of A) nitrogen stable isotope ratios (δ^{15}N) and B) total N content (% N) in *Cystoseira mediterranea* tissue after 24 h of exposure to 70-, 100-, 130-, 160-, and 200- μM concentrations of NH_4^+. Dotted line represents initial δ^{15}N and % N content.

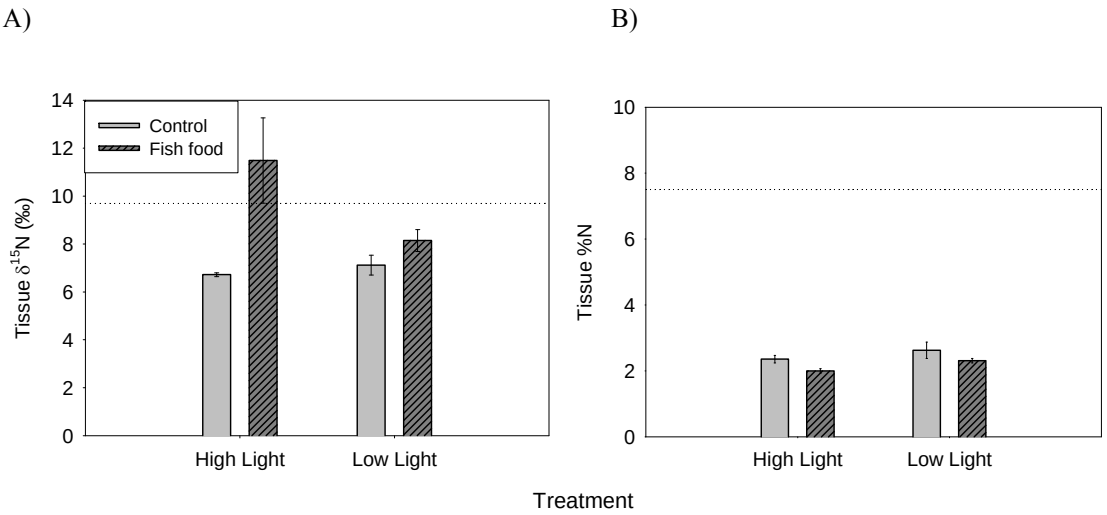


Figure 3: Mean values of A) nitrogen stable isotope ratios ($\delta^{15}N$) and B) total N content (% N) in *Cystoseira mediterranea* tissue exposed to control and fish food treatments (n=3) at different light conditions (High Light and Low Light conditions). Dotted line represents $\delta^{15}N$ and % N content in fish food.

Discussion

The results obtained in the experiments presented here indicated that *Cystoseira mediterranea* do not present selection for ^{14}N versus ^{15}N atoms (fractionation) under varying nutrient features (ratios of $\delta^{15}N$) and nutrient availability (concentrations of N) conditions.

Neither of our experiments (experiments 1 and 2) provided evidence that ^{14}N was preferred or selected over ^{15}N by *C. mediterranea* across the range of ratios of $\delta^{15}N$ and concentrations of N tested. Although diatoms demonstrated strong preferences for ^{14}N under conditions when N was not limiting (Pennock et al., 1996), our results suggest that *C. mediterranea* does not fractionate even when N supply is sufficient, as in the experiment 1.

Rather, *C. mediterranea* takes up ^{14}N and ^{15}N in direct proportion to supply in the source, suggesting that the alga may be useful for determining N sources in ecological studies. On the other hand, our results do not support concentration-dependent fractionation for *C. mediterranea* as has frequently been demonstrated for phytoplankton (Wada and Hattori, 1978; Cifuentes et al. 1989; Pennock et al. 1996). Instead, in the experiment 2, algae took up ^{15}N in the same proportion across a wide range of concentrations. This agrees with others works studying other species of macroalgae (Cohen and Fong, 2005).

Regards the results obtained varying light conditions we observed that δ^{15}N in macroalgal tissues respond to alterations in light levels (experiment 3). We found higher δ^{15}N values in plant incubated under high light conditions than in those incubated in low light conditions. Other studies found that plants that grown under low light conditions are isotopically depleted in heavier isotopes compared to plants from high light environments (Grice et al., 1996; Vizzini et al., 2003). So, light availability may lead to differential fractionation of the available isotope pools. The apparent positive discrimination for δ^{15}N for ammonium in the light treatment is within the range of previous studies conducted on higher plants and microalgae (Wada and Hattori, 1978; Pennock et al., 1996; Waser et al., 1998a). Despite macroalgal uptake is controlled by light availability (Mann, 1982) we do not found differences in total N content between plants incubated in high and low light conditions. So, the quantity of nitrogen absorbed by the plant is not the responsible of differences in δ^{15}N values.

Effects of light availability on fractionation in macroalgae may be also mediated by other factors such as the source of nitrogen available to the plant. Dudley (2007) found that the effect of light reduction on fractionation during growth is dissimilar for nitrate and ammonium suggesting a strong relationship between light reduction and fractionation for ammonium-fed and a weak relationship between light reduction and fractionation during growth on nitrate. It was likely to the capacity of the studied alga *Ulva* sp. to store nitrate in unreduced form (Naldi and Wheeler 1999) that may result in low efflux of δ^{15}N -enriched internal pools of unreduced

nitrate back into the external medium from *Ulva* sp. tissue even when nitrogen is supplied in excess to growth requirements as in the shaded conditions.

Our results also demonstrated experimentally that the macroalgal isotopic composition is a good indicator of N derived from fish farms (experiment 3) similar to others studies have demonstrated with field measurements (Jones et al., 2001; Vizzini and Mazzola, 2004; Pérez et al., 2008; García-Sanz et al., in press). Macroalgae carry the history of the nutrients to which they were exposed for weeks, unlike the nutrients in phytoplankton that are completely turned over in a few days. So macroalgae could be good indicators of the spatial extent of dispersal of dissolved nitrogen derived from fish farms in offshore waters where is difficult to detect nutrient enrichment by means of standard physicochemical analyses due to the high hydrodynamic nature of this environment (Karakassis et al., 2001; Sarà, 2007). Our results show how macroalgae reflects the isotopic signal of the wastes derived from fish farm (fish food), but only in high light conditions. Then, possible variations in $\delta^{15}N$ signatures due to variations in light intensity must be taken into account in the design of studies using $\delta^{15}N$ signatures to infer sources of nutrient enrichment since $\delta^{15}N$ in macroalgal tissue differs between macroalgae tissue sampled or incubated near the surface and at depth (Cornelisen et al., 2007; García-Sanz et al., in press).

This work helps to illuminate some of the physiological mechanisms that may be important in fractionation of nitrogen during its assimilation into macroalgae, and have strong implications for a wide range of stable isotope approaches that rely on understanding of uptake of nitrogen isotopes to interpret nutrient and/or trophic pathways through ecological systems. One general consequence of these results concerns the application of $\delta^{15}N$ ratios to infer patterns of eutrophication. Values of $\delta^{15}N$ in study organisms are often evaluated over environmental gradients that can include factors that may co-vary with concentrations of enriched nitrogen. As a result, interpretation of patterns of nutrient enrichment may be confounded by environmental gradients (e.g., light or nutrient concentration) that affect the

transfer of δ^{15}N in organisms such as *Cystoseira mediterranea*. This study provides direct experimental evidences demonstrating that macroalgae did not exhibit N isotope fractionation under different ratios of δ^{15}N and concentrations of N. However, light intensity must be taken into account since δ^{15}N ratios respond to alterations in light levels. A macroalgal indicator would work best either in steady-state environments or as a bioassay where the algae is first cultured and then outplanted or incubated to the field. At this point, we can use macroalgae to detect differences in δ^{15}N signatures at different locations by comparing them with one another but cannot deduce absolute δ^{15}N values of the water column from tissue δ^{15}N values without further studies investigating macroalgal fractionation.

DISCUSIÓN GENERAL Y CONCLUSIONES

Discusión general

La investigación desarrollada en este trabajo, por una lado, ha aportado información sobre las respuestas fisiológicas de la fanerógama marina *Posidonia oceanica* a los vertidos procedentes de granjas marinas (Capítulo I) a la vez que ha demostrado la utilidad de algunas variables fisiológicas, y en especial de la señal de $\delta^{15}N$ en los tejidos de las fanerógamas marinas (*Posidonia oceanica* y *Cymodocea nodosa),* como indicadora del destino de los aportes de N disuelto derivado de los vertidos de acuicultura en estos ecosistemas (Capítulo II). Por otro lado, se ha desarrollado y aplicado un método experimental para determinar el alcance espacial en la columna de agua de dichos vertidos basado en el análisis del $\delta^{15}N$ en macroalgas mediante el empleo de bioensayos (Capítulos III y IV). Por último, se ha demostrado experimentalmente la eficacia del $\delta^{15}N$ de las macroalgas para reflejar el nitrógeno procedente de los vertidos de las granjas marinas bajo diferentes condiciones ambientales de concentración de nitrógeno y de luz (Capítulo V). Dado que los resultados obtenidos ya han sido discutidos exhaustivamente en los capítulos correspondientes, en este apartado vamos a presentar una discusión más general sobre el potencial de los distintos indicadores aquí desarrollados y de las diversas aplicaciones que podrían tener para contribuir a mejorar la gestión de las granjas marinas en lo que se refiere, especialmente al impacto derivado de los nutrientes de sus efluentes.

Las fanerógamas marinas y la acuicultura marina: impactos y respuestas.

Los hábitats constituidos por fanerógamas marinas tienen una gran importancia ecológica debido a los beneficios y servicios que prestan al ecosistema (elevada producción primaria, control de la calidad del agua, sedimentación, biodiversidad) ampliamente reconocidos por la comunidad científica internacional (Larkum et al., 2006). De hecho, estos hábitats se encuentran expresamente protegidos por la Unión Europea en base a la Directiva 92/43/CEE, de 21 de mayo, del Consejo, relativa a la conservación de los hábitats naturales y de la fauna y flora

silvestres, por la cual las praderas de fanerógamas marinas figuran como Lugares de Interés Comunitario (LIC).

Las zonas costeras del Mediterráneo están en gran parte ocupadas por praderas de la fanerógama marina *Posidonia oceanica* (Green y Short 2003). Esta especie es endémica de este mar y sus praderas ocupan grandes extensiones distribuyéndose entre los 0.5 y 30 m de profundidad y cubriendo un área de 25.000 a 50.000 km^2, lo que representa un 1-2 % de la superficie del mar Mediterráneo (Pasqualini et al., 1998). Por otra parte, *Cymodocea nodosa* es otra especie de fanerógama marina que podemos encontrar formando praderas en el litoral Mediterráneo. Esta especie, de origen tropical, se encuentra actualmente restringida al Mediterráneo y al Atlántico nororiental, desde el sur de Portugal hasta Senegal, incluyendo las islas Canarias y Madeira (Marbà y Terrados, 2004). Su rango batimétrico de distribución va desde 1 a 2 metros en zonas muy abrigadas, hasta los 35-40 metros de profundidad (Reyes et al., 1995).

El desarrollo de la industria acuícola, en particular de la acuicultura marina en granjas *off-shore* ha generado un conflicto de intereses entre la ocupación del espacio marino por parte de los acuicultores y una adecuada conservación de los ecosistemas de fanerógamas marinas. Esto se debe a que las granjas marinas *off-shore* han de situarse a una distancia prudencial de los puertos para que su explotación sea económicamente rentable. Por lo tanto, estas granjas se suelen situar a una distancia de entre 1 y 3 km de la costa, con profundidades que varían de 20 a 45 metros, zona que coincide con el área de distribución de las praderas de fanerógamas. Actualmente en España se están elaborando planes de ordenación de espacios aptos para la instalación de granjas marinas, sin embargo tan solo cuatro comunidades (Cataluña, Andalucía, Galicia y Murcia) han regulado en detalle la creación de zonas de interés de cultivos marinos. Estos planes consideran incompatible el establecimiento de instalaciones acuícolas con la presencia de comunidades de fanerógamas marinas. Sin embargo, aunque esté expresamente

prohibido instalar granjas marinas sobre estos ecosistemas, los vertidos derivados de estas instalaciones pueden afectar a praderas que se encuentren en las inmediaciones de las mismas.

Las praderas de *Posidonia oceanica* son muy vulnerables al enriquecimiento de nutrientes derivado de los vertidos de las granjas marinas (Delgado et al., 1997; Ruiz et al., 2001; Pergent-Martini et al., 2006; Homer et al., 2008). Por lo tanto, la búsqueda de indicadores tempranos (*early-warnig*) que permitan determinar la influencia de los vertidos antes de que estos produzcan efectos irreversibles en las plantas revierte especial importancia en la monitorización de estos ecosistemas. En este sentido, este trabajo demuestra que determinadas variables fisiológicas muestran respuestas en las praderas expuestas a los vertidos con respecto a las praderas control. Entre las variables fisiológicas que mejor respuesta obtuvieron se encuentran el contenido total de N y $\delta^{15}N$ en epífitos, el contenido total de P tanto en epífitos como en rizomas, el contenido total de TNC y la concentración de FAA en rizomas así como la composición específica de FAA tanto en rizomas como en raíces. Por lo tanto, estas variables fisiológicas se proponen para su uso como herramientas de gestión para monitorizar y poder detectar una degradación ambiental en las praderas cercanas a instalaciones de acuicultura.

Una de las ventajas del uso de indicadores basados en la fisiología de la planta con respecto a otras herramientas que se usan rutinariamente en la monitorización de estos ecosistemas (e.g. seguimiento de variables demográficas) es que pueden responder con rapidez ante cambios en los aportes de nutrientes. Como desventaja podríamos señalar la posible interferencia de otros factores, como el grado de anoxia del sedimento (Pérez et al., 2007), en la respuesta de alguna de las variables fisiológicas citadas, debido la compleja regulación del metabolismo en las angiospermas marinas (Touchette y Burkholder, 2000).

Otro aspecto importante a tener en cuenta para poder compatibilizar el desarrollo de la acuicultura en granjas *off-shore* y la conservación de los ecosistemas de fanerógamas marinas es la denominada “distancia de seguridad” a ecosistemas sensibles, es decir la distancia que debe

mediar entre la instalación marina y el inicio de la pradera para asegurar que no se producirá ningún impacto negativo sobre la misma. Estudios recientes que usan nuevas técnicas de muestreo para monitorizar estos ecosistemas (Holmer et al., 2008) recomiendan una distancia de seguridad entre las granjas y el inicio de la pradera de 400 m que es dos veces mayor a la distancia de 200 m sugerida por Pergent-Martini et al., (2006) en una revisión de datos existentes usando indicadores menos sensibles, tanto abióticos (luz, sedimento, agua intersticial) como bióticos (densidad de la pradera, lepidocronología, producción primaria, etc.). En nuestro estudio, se obtuvieron distancias de seguridad mayores a las mencionadas mediante el uso del $\delta^{15}N$ como indicador de la dispersión de N disuelto derivado de vertidos de acuicultura. Con este método, el nitrógeno procedente de las granjas marinas se detectó a distancias que van desde los 200 m (caso de la granja de Cataluña) hasta más de 2000 m (en el caso del complejo de granjas de la zona de Murcia). Sin embargo, hay que tener en cuenta que este indicador informa sobre si las praderas se encuentran bajo la influencia del vertido pero no sobre si este vertido está produciendo impactos negativos en la pradera. Nuestros resultados también demostraron que el análisis de $\delta^{15}N$ es más sensible que el contenido total de N (usado más habitualmente) a la hora de detectar si el nitrógeno derivado de las granjas marinas está llegando a las praderas cercanas a las instalaciones lo que permite ampliar las distancias de seguridad antes mencionadas y poder delimitar el radio de influencia de los vertidos antes de que se produzcan efectos negativos significativos en las praderas.

Por último cabe destacar que los resultados obtenidos en estos estudios apuntan a los rizomas (principal órgano de almacenaje) y los epífitos (más efectivos a la hora de captar nutrientes que la planta y con procesos fisiológicos más simples) como los compartimentos más sensibles a cambios asociados con la entrada de nutrientes procedentes de granjas marinas. En cambio, los tejidos fotosintéticos no respondieron tan bien como cabía esperar a pesar de que varios autores han constatado que estos órganos responden positivamente al enriquecimiento en N (Touchette et al., 2003; Invers et al., 2004; Leoni et al., 2007) y de que es el compartimento más ampliamente utilizado en estudios de enriquecimiento de nutrientes.

Bioensayos con macroalgas como indicadores de la dispersión del N procedente de granjas marinas.

Las granjas marinas constituyen importantes focos de liberación de residuos en el medio circundante. Dichos residuos son tanto particulados (restos de alimento no ingerido, heces, organismos muertos) como disueltos (nutrientes orgánicos e inorgánicos). Los residuos particulados se depositan en el sedimento pudiendo alterar sus condiciones fisicoquímicas y la macrofauna asociada al mismo (Karakassis et al., 2000). Estos residuos y sus impactos asociados se detectan en un radio generalmente no superior a 30-100 m de la granja marina, aunque esta distancia varía principalmente en función de la profundidad del fondo, el hidrodinamismo y el tipo de sedimento (Brooks y Manhken, 2003; Kalantzi y Karakassis, 2006). Por otro lado, el destino de los residuos disueltos es más difícil de detectar que el de los residuos particulados ya que el material disuelto se dispersa en la columna de agua haciendo difícil su identificación. Además, en mares oligotróficos como el Mediterráneo, los aportes de nutrientes procedentes de las granjas marinas pueden unirse con los procedentes de otras fuentes (ríos, aguas residuales, etc.) sumando sus efectos y haciendo aun más complicada su identificación.

Conocer el destino y efectos de los residuos disueltos en la columna de agua es esencial para poder tomar medidas adecuadas orientadas a una gestión sostenible de las granjas marinas. En este sentido, una de las principales aportaciones de esta tesis ha sido el desarrollo y evaluación de un método para detectar el alcance espacial del nitrógeno disuelto en la columna de agua procedente de vertidos marinos de la acuicultura en aguas costeras basado en el análisis de la señal isotópica del N ($\delta^{15}N$) en bioensayos con macroalgas. Este método solventa las carencias que existen a la hora de detectar nutrientes derivados de las instalaciones de acuicultura en la columna de agua, ya que a pesar de la cantidad, relativamente elevada de nutrientes liberados al medio marino por las granjas marinas, la mayoría de los estudios

realizados con métodos tradicionales no detectan un aumento significativo de las concentraciones de nutrientes en el agua cerca de las jaulas (La Rosa et al., 2002; Soto y Norambuena, 2004; Pitta et al., 1999, 2006; Sara, 2007). Además dado que en muchos casos, la vegetación bentónica no está presente en las proximidades de las jaulas de cultivo y que por lo tanto no puede utilizarse como bioindicador en todos los casos, el uso de bioensayos aparece como una alternativa viable para solventar las carencias que existen a la hora de detectar el destino de los nutrientes derivados de granjas de acuicultura en la columna de agua.

Los bioensayos desarrollados en este estudio, consistentes en incubar fragmentos de macrófitos a diferentes distancias de las jaulas y analizar el $\delta^{15}N$ de los mismos, han permitido constatar que la distancia de detección del vertido obtenida en las tres granjas marinas estudiadas fue diferente en función de diversos factores tales como el tamaño de la granja, la gestión de la misma, la especie cultivada, la producción del cultivo y la hidrografía y batimetría de la zona, pero, en general, fue superior a las distancias obtenidas en granjas con características similares aplicando otros métodos de detección menos sensibles (Pergent et al., 2006; Sarà 2007). En la granja de las Islas Canarias la distancia de detección del vertido fue de 450-700 m. En la zona de Murcia fue mayor (1550-2450 m) debido al elevado volumen de producción, no solamente de la granja de atunes estudiada, sino del conjunto de granjas marinas situadas en el complejo de San Pedro del Pinatar. Este resultado pone en evidencia el potencial de este método como herramienta de gestión fiable y eficaz para determinar la influencia real de complejos acuícolas que ocupan grandes extensiones y cuya influencia sobre el medio no puede ser evaluada a partir de la suma de vertidos individuales. Por último, la distancia de influencia del vertido en Cataluña fue como máximo de 120 m siendo esta distancia menor que la esperada en función de la producción de la granja probablemente debido a la influencia de otras fuentes de nitrógeno que podrían derivar de los aportes procedentes de la desembocadura del río Ebro. Estos aportes tienen una señal elevada de $\delta^{15}N$, lo que hace que la señal del medio también sea elevada y que los bioensayos con macroalgas no sean tan sensibles a la hora de detectar nitrógeno procedente de las granjas.

Aplicación de los bioensayos a la gestión sostenible de la acuicultura marina en jaulas.

La aplicación del método de bioensayos puede contribuir a mejorar la gestión de las granjas marinas aumentando con ello la sostenibilidad de esta industria en expansión. Uno de los requisitos principales para una práctica sostenible de la acuicultura consiste en disponer de criterios para poder seleccionar las zonas más adecuadas para el desarrollo de esta actividad. Cualquier plan de zonificación en acuicultura debe incluir una delimitación espacial lo más precisa posible del vertido procedente de las instalaciones de acuicultura. Para ello, las metodologías utilizadas (SIG, modelos dinámicos, imágenes de satélite, etc.) se pueden complementar con la información aportada por la aplicación del método de bioensayos dado que este método estima el radio de influencia de los vertidos procedentes de granjas marinas con gran precisión y proporciona distancias de seguridad mayores que las obtenidas con otras técnicas.

También, el uso de bioensayos se puede combinar con nuevos modelos que se están desarrollando para evaluar el impacto de las granjas marinas en el bentos tales como el modelo DEPOMOD validado para el salmón en Escocia (Cromey et al., 2002) y el modelo MERAMOD utilizado para la dorada y la lubina en el Mediterráneo (Cromey et al., 2004). Estos modelos predicen los efectos en las comunidades bentónicas en respuesta a modelos de deposición de sólidos procedentes de granjas marinas y han incorporado recientemente la resuspensión de sedimentos como un factor relevante a la hora de entender la dinámica de los vertidos (Dudley et al., 2000). La aplicación del método de los bioensayos puede ayudar a detectar los procesos de resuspensión de sedimentos de manera eficaz por lo que la combinación de ambos métodos podría resultar efectiva para obtener información de los efectos de los vertidos tanto en el bentos como en la columna de agua.

Otro de los aspectos principales para aumentar la sostenibilidad de las actividades acuícolas consiste en reducir la carga de nutrientes de sus efluentes a través de una apropiada gestión de las granjas marinas. Como el mayor aporte de materia orgánica y de nutrientes al medio proviene de los procesos de alimentación y metabolismo de los peces, la composición y calidad de las dietas y las prácticas de alimentación son aspectos claves a la hora de gestionar mejor los residuos de una granja marina. Estudios recientes recomiendan mejorar la composición de las dietas eliminado ingredientes de digestibilidad baja (tales como cereales integrales o subproductos a base de cereales usados como ligandos) e incorporando ingredientes de digestibilidad alta con buenas propiedades para ligar los componentes del pienso (Schneider et al., 2004). Además se ha propuesto sustituir aceites y componentes animales en la dieta por componentes vegetales tales como la soja o el maíz (Bell y Waagbø 2008). Con la aplicación de este tipo de medidas (entre otras) se puede reducir la *food conversion rate* y minimizar el vertido de materia orgánica en el medio. Por otra parte, se pueden mejorar las prácticas de alimentación mediante sistemas automatizados de suministro de piensos que repartan lo más homogéneamente posible el alimento en el sistema de cultivo además de fomentar que se mantengan unas condiciones adecuadas de almacenamiento de las dietas para asegurar la calidad nutricional y palatabilidad del alimento. Estas medidas también consiguen reducir la carga de nutrientes que se liberan al medio de manera significativa. En este sentido, la monitorización de los residuos de las granjas marinas mediante el método de los bioensayos y del análisis de las señal $\delta^{15}N$ en las algas incubadas aportaría información relevante sobre la efectividad de las medidas de gestión relacionadas con cambios en la composición de las dietas y cambios en las estrategias de alimentación destinadas a reducir la carga de nutrientes en el medio.

Otras prácticas recomendadas para una gestión sostenible de las granjas marinas que minimizan o reducen el impacto de los efluentes de las mismas son los sistemas biológicos que actúan como filtros (biofiltros) de la materia orgánica y nutrientes (Midlen y Redding, 1998) o los sistemas integrados de cultivo (policultivos) basados en la cría conjunta de más de una

especie en los que los residuos de una especie se utilizan como fuente de alimento para otras especies, aprovechando y revalorizando la materia orgánica, reduciendo el volumen total de residuos de la instalación y aumentando la producción total de biomasa (Neori et al., 2004; Schneider et al., 2005). Diferentes estrategias basadas en este tipo de enfoques se han utilizado durante muchos años en Asia, incluyendo China y se están desarrollando en numerosos países en la actualidad (Alongi et al., 2000). Sin embargo, la aplicación de este tipo de prácticas en granjas marinas *off-shore* resulta difícil aunque actualmente se están realizando estudios para poder aplicar estos modelos a estos tipos de granjas. Por ejemplo, algunos autores (Angel y Spanier, 2002) han recomendado la construcción de estructuras físicas en los alrededores de las granjas marinas para promover la aparición de comunidades vegetales y de animales filtradores que retengan y utilicen la materia orgánica para su supervivencia. Otros autores (Lupatsch et al., 2003) han sugerido el cultivo de peces demersales para eliminar parte de la materia orgánica acumulada en el sedimento. En todos estos casos, el seguimiento del destino de los nutrientes y la eficacia en la captación de los mismos tanto en sistemas de biofiltros como en policultivos puede realizarse mediante el método del análisis de la señal isotópica del N, pudiendo resultar efectivo para comprobar si realmente esos cultivos minimizan los vertidos o para darnos una idea de la eficiencia en la transferencia de N de un cultivo a otro ya que, como se ha visto en esta tesis, la señal isotópica del nitrógeno es un buen indicador del nitrógeno proveniente de las granjas.

El desarrollo de indicadores tales como los que hemos presentado a lo largo de esta tesis resulta especialmente importante debido a que la mayor parte de la acuicultura en jaulas tiene lugar en el medio ambiente costero, ya de por si frágil y sometido a una gran presión, por lo que existe una necesidad cada vez mayor de dar un empuje a la sostenibilidad medioambiental de este subsector de la industria acuícola para hacer que esta actividad sea compatible con la conservación de los sistemas costeros marinos dado que estos sustentan una compleja interacción de ecosistemas distintos, con una enorme biodiversidad, y se encuentran entre los más productivos del mundo. Sin embargo, cualquier mejora en la gestión de los vertidos

procedentes de las instalaciones de acuicultura debería de ir acompañada de cambios en las políticas de gestión de los recursos tales como la reducción de la piscicultura de peces de alto nivel trófico (Stergiou et al., 2009) ya que este tipo de acuicultura implica que se ejerza una importante presión pesquera sobre los peces pelágicos pequeños para hacer piensos y como no está previsto que las existencias de pequeños pelágicos aumenten en el futuro, la expansión de la acuicultura quedaría limitada, cuando menos si concebimos la acuicultura como la cría de peces carnívoros, que es como la entendemos generalmente en los países occidentales.

Conclusiones

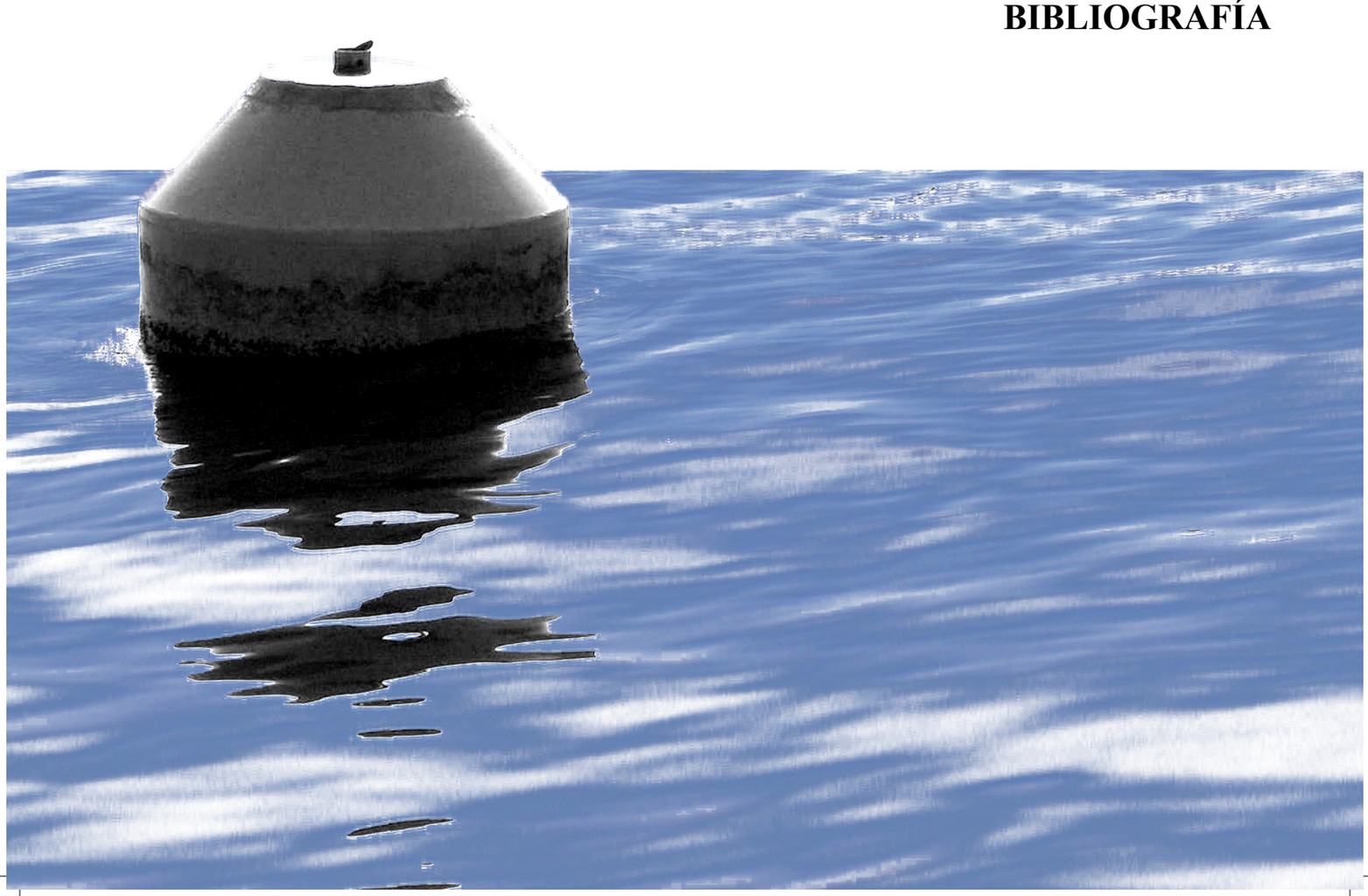
A continuación se recogen las principales conclusiones derivadas de esta tesis y que ya han sido comentadas en cada capítulo de manera independiente.

1. La exposición a vertidos procedentes de granjas marinas influye en determinadas variables fisiológicas (% N, $\delta^{15}N$, % P, TNC y FAA) la de la fanerógama marina *P. oceanica* y sus epífitos por lo que se recomiendan dichas variables como indicadoras del impacto de granjas marinas en estos ecosistemas.
2. El análisis de $\delta^{15}N$ en fanerógamas marinas refleja mejor el régimen externo de nutrientes que el contenido total de N y pone de manifiesto la influencia remota de los vertidos de granjas marinas sobre las praderas de fanerógamas estudiadas ampliando las distancias de seguridad recomendadas en estudios anteriores a un rango entre 200 y 2000 m dependiendo del tamaño de la instalación.
3. El $\delta^{15}N$ medido en macroalgas incubadas en bioensayos muestra un claro y significativo incremento respecto a los valores control a medida que la distancia entre los bioensayos y las jaulas de peces disminuye, demostrando el potencial y eficacia del método de bioensayos con macroalgas para identificar gradientes de nutrientes y caracterizar de forma eficiente el alcance y la dirección de los vertidos procedentes de las diferentes granjas estudiadas. Los resultados obtenidos en la evaluación del método de bioensayos indican que:
    a. La intensidad de la señal isotópica es especie-específica, dependiendo de la afinidad de los macrófitos por asimilar nutrientes externos.
    b. La intensidad de la señal es más evidente en la capa superficial (-5 m) que en la profunda (-20 m), probablemente debido a una combinación de factores relativos al funcionamiento del cultivo (alimentación en superficie) y oceanográficos (formación de termoclina), que redujeron el alcance de los aportes orgánicos a las capas profundas en la época de estudio (verano).

c. Un tiempo de incubación de 4-6 días es suficiente para obtener una buena caracterización de los gradientes espaciales de los aportes orgánicos procedentes de los cultivos.

4. Las distancias máximas de detección de vertidos procedentes de granjas marinas utilizando el método de bioensayos fueron de entre 200 y 2000 m dependiendo del sitio de estudio y de las características de las granjas marinas. Estas distancias de detección fueron mayores en una dirección que en otra dependiendo de la dirección de los vientos dominantes en el momento de la experiencia poniendo en evidencia el carácter anisotrópico de la dispersión de los vertidos acuícolas.
5. El uso de bioensayos para monitorizar nutrientes procedentes de granjas marinas se ve apoyado por los resultados obtenidos en los experimentos de laboratorio realizados en los que *Cystoseira mediterranea* incrementa su señal al estar expuesta a piensos de peces y en los que no existe fraccionamiento dependiente de la concentración o del cociente isotópico. La variabilidad en el fraccionamiento entre el ^{14}N y ^{15}N encontrada en *C. mediterranea* en función de los niveles de luz durante su incubación apunta a la necesidad de tomar en cuenta este factor en el diseño de evaluaciones del impacto que usen el $\delta^{15}N$ como trazador del flujo de nitrógeno.

BIBLIOGRAFÍA



Bibliografía

Aguado-Giménez, F., García-García, B., 2005. Growth, food intake and feed conversion rates in captive Atlantic bluefin tuna (*Thunnus thynnus* Linnaeus, 1758) under fattening conditions. Aquaculture Research 36, 610-614.

Aguado-Giménez, F., García-García, B., Hernández-Lorente, M.D., Cerezo-Valverde, J., 2006. Gross metabolic waste output estimates using a nutritional approach in Atlantic bluefin tuna (*Thunnus thynnus*) under intensive fattering conditions in western Mediterranean Sea. Aquaculture Research 37, 1254-1258.

Aguado-Giménez, F., Marín A., Montoya, S., Marín Guirao, L., Piedecausa, A., García-García, B., 2007. Comparison between some procedures for monitoring offshore cage culture in western Mediterranean Sea: Sampling methods and impact indicators in soft substrata. Aquaculture 271, 357–370.

Alamoudi, O.A., 1994. Species-differences in nitrogen storage reservoirs in macroalgae. Microbios 77, 239-246.

Albayrak, S., Balkis, H., Zenetos, A., Kuruna, A., Kubanç, C., 2006. Ecological quality status of coastal benthic ecosystems in the Sea of Marmara. Marine Pollution Bulletin 52, 790-799.

Alcoverro, T., Manzanera, M., Romero, J., 2001. Annual metabolic carbon balance of the seagrass *Posidonia oceanica*: the importance of carbohydrate reserves. Marine Ecology Progress Series 211, 105-116.

Alcoverro, T., Zimmerman, R.C., Kohrs, D.G., Alberte, R.S., 1999. Resource allocation and sucrose mobilization in light-limited eelgrass *Zostera marina*. Marine Ecology Progress Series 187, 121-131.

Alongi, D.M., Chong, V.C., Dixon, P., Sasekumar, A., Tirendi, F., 2003. The influence of fish cage aquaculture on pelagic carbon flow and water chemistry in tidally dominated

mangrove estuaries of peninsular Malaysia. Marine Environmental Research 55, 313-333.

Alongi, D.M., Johnston, D.J., Xuan, T.T., 2000. Carbon and nitrogen budgets in shrimp ponds of extensive mixed shrimp– mangrove forestry farms in the Mekong Delta, Vietnam. Aquaculture Research 31, 387– 399.

Angel, D.L., Spanier, E., 2002. An application of artificial reefs to reduce organic enrichment caused by net cage fish farming: preliminary results. ICES Journal of Marine Science 59, 1-6.

Apostolaki, E.T., Marbà, N., Holmer, M., Karakassis, I., 2009. Fish farming enhances biomass and nutrient loss in *Posidonia oceanica* (L.) Delile. Estuarine Coastal and Shelf Science 81, 390-400.

Apostolaki, E.T., Tsagaraki, T., Tsapakis, M., Karakassis, I., 2007. Fish farming impact on sediments and macrofauna associated with seagrass meadows in the Mediterranean. Estuarine, Coastal and Shelf Science 75, 408-416.

Aquaetreat, 2007. Manual on effluent treatment in Aquaculture: Science and Practice. 163 pp. (www.aquaetreat.org).

Aravena, R., Evans, M.L., Cherry. J.A., 1993. Stable isotopes of oxygen and nitrogen in source identification of nitrate from septic systems. Ground Water 31, 180-186.

Ballesteros, E., 1988. Estructura y dinámica de la comunidad de *Cystoseira mediterranea* Sauvageau en el Mediterráneo Noroccidental. Investigación Pesquera 52, 313–334.

Ballesteros, E., Pérez, M., Zabala, M., 1984. Aproximación al conocimiento de las comunidades algales de la zona infralitoral superior en la costa catalana. Collectanea Botanica 15, 69–100.

Ballesteros, E., Torras, X., Pinedo, S., García, M., Mangialajo, L., de Torres, M., 2007. A new methodology based on littoral community cartography dominated by macroalgae for the implementation of the European Water Framework Directive. Marine Pollution Bulletin 55, 172-180.

Basurco, B., Larrazabal, G., 2000. Marine fish farming in Spain. Cahiers options méditerranéennes 30, 45-56.

Basurco, B., Lovatelli, A., 2003. The aquaculture situation in the Mediterranean Sea. Predictions for the future. Extracted from the International Conference on the Sustainable Development of the Mediterranean and Black Sea Environment. (http://www.iasonnet.gr/).

Bell, G.J., Waagbø, R. 2008. Safe and nutritious aquaculture produce: benefits and risks of alternative sustainable aquafeeds. In Holmer M, Black K, Duarte CM, Karakassis I, Marbá N (eds.) Aquaculture in the Ecosystem. Springer, Netherlands, p. 185-226

Bellan-Santini, D., 1968. Influence de la pollution sur les peuplements benthiques. Revue International d'Oceanographie Méditerranéenne 10, 27–53.

Beveridge, M., 2004. Cage Aquaculture, third edition. Oxford, UK, Blackwell Publishing Ltd. 368 pp.

Beveridge, M.C.M., Ross L.G., Kelly, L.A., 1994. Aquaculture and biodiversity. Ambio 23, 497-502.

Bokn, T.L., Moy, F.E., Christie, H., Engelbert, S., Karez, R., Kersting, K., Kraufvelin, P., Lindblad, C., Marba, N., Pedersen, M.F., Sorensen., K., 2002. Are rocky shore ecosystems affected by nutrient-enriched seawater? Some preliminary results from a mesocosm experiment. Hydrobiologia 484, 167-175.

Borja, A., Franco, J., Pérez, V., 2000. A marine biotic index to establish the ecological quality of soft-bottom benthos within european estuarine and coastal environments. Marine Pollution Bulletin 40, 1110–1114.

Borum, J., 1985. Development of epiphytic communities on eelgrass (*Zostera marina*) along a nutrient gradient in a Danish estuary. Marine biology 87, 211-218.

Bowen, J.L., Valiela, I., 2008. Using delta N-15 to assess coupling between watersheds and estuaries in temperate and tropical regions. Journal of Coastal Research 24, 804-813.

Brooks, K.M., Mahnken, C.V.W., 2003. Interactions of Atlantic salmon in the pacific northwest environment II. Organic wastes. Fisheries Research 62, 255-293.

Burford, M.A., Costanzo, S.D., Dennison, W.C., Jackson, C.J., Jones, A.B., McKinnon, A.D., Preston, N.P., Trott, L.A., 2003. A synthesis of dominant ecological processes in

intensive shrimp ponds and adjacent coastal environments in NE Australia. Marine Pollution Bulletin 46, 1456-1469.

Burkholder, J.M., Tomasko, D., Touchette, B.W., 2007. Seagrasses and eutrophication. Journal of Experimental Marine Biology and Ecology 350, 46-72.

Cancemi, G., De Falco, G., Pergent, G., 2003. Effects of organic matter input from a fish farming facility on a *Posidonia oceanica* meadow. Estuarine, Coastal and Shelf Science 56, 961-968.

Cardia, F., Lovatelli, A., 2007. A review of cage aquaculture: Mediterranean Sea. In M. Halwart, D. Soto and J.R. Arthur (eds). Cage aquaculture – Regional reviews and global overview, pp. 156–187. FAO Fisheries Technical Paper. No. 498. Rome, 241 pp.

Christensen, P.B., Rysgaard, S., Sloth,N.P., Dalsgaard, T., Schwærter, S., 2000. Sediment mineralization, nutrient fluxes, denitrification and dissimilatory nitrate reduction to ammonium in an estuarine fjord with sea cage trout farms. Aquatic Microbial Ecology 21, 73-84.

Chua, T.E., 1992. Coastal aquaculture development and the environment: The role of coastal area management. Marine Pollution Bulletin 25, 98-103.

Cifuentes, L.A., Fogel, M.L., Pennock, J.R., Sharp, J.H., 1989. Biogeochemical factors that influence the stable nitrogen isotope ratio of dissolved ammonium in the Delaware estuary. Geochimica et Cosmochimica Acta 53, 2713-21.

Cifuentes, L.A., Sharp, J.H., Fogel, M.L., 1988. Stable carbon and nitrogen isotope biogeochemistry in the Delaware estuary. Limnology and Oceanography 33, 1102-15.

Cohen, R.A., Fong, P., 2005. Experimental evidence supports the use of $\delta^{15}N$ content of the opportunistic green macroalga *Enteromorpha intestinalis* (Chlorophyta) to determine nitrogen sources to estuaries. Journal of Phycology 41, 287-293.

Cole, M.L., Kroeger, K.D., McClelland, J.W., Valiela, I., 2005. Macrophytes as indicators of land-derived wastewater: Application of a $\delta^{15}N$ method in aquatic systems. Water Resources Research 41, W01014.

Cole, M.L., Valiela, I., Kroeger, K.D., Tomasky, G.L., Cebrian, J., Wigand, C., McKinney, R.A., Grady, S.P., Carvalho da Silva, M.H., 2004. Assessment of a $\delta^{15}N$ Isotopic Method to Indicate Anthropogenic Eutrophication in Aquatic Ecosystems. Journal of Environmental Quality 33, 124-132.

Cornelisen, C.D., Wing, S.R., Clark, K.L., Bowman, M.H., Frew, R.D., Hurd, C.L., 2007. Patterns in the $\delta^{13}C$ and $\delta^{15}N$ signature of *Ulva pertusa*: Interaction between physical gradients and nutrient source pools. Limnology and Oceanography 52, 820-832.

Costanzo, S.D., O'Donohue, M.J., Dennison, W.C., 2000. *Gracilaria edulis* (Rhodophyta) as a biologycal indicador of pulsed nutrients in oligotrophic waters. Journal of Phycology 36, 680-685.

Costanzo, S.D., O'Donohue, M.J., Dennison, W.C., 2003. Assessing the seasonal influence of sewage and agricultural nutrient inputs in a subtropical river estuary. Estuaries 26, 857–865.

Costanzo, S.D., O'Donohue, M.J., Dennison, W.C., Loneragan, N.R., Thomas, M., 2001. A new approach for detecting and mapping sewage impacts. Marine Pollution Bulletin 42, 149-156.

Costanzo, S.D., O'Donohue, M.J., Dennison, W.C., 2004. Assessing the influence and distribution of shrimp pond effluent in a tidal mangrove creek in north-east Australia. Marine Pollution Bulletin 48, 514-525.

Cromey, C., Nickell, T., Black, K., Provost, P., Griffiths, C., 2002. Validation of a fish farm waste resuspension model by use of a particulate tracer discharged from a point source in a coastal environment. Estuaries 25, 916-929.

Cromey, C.J., Black, K.D., Blackstock, J., Carroll, M., Karakassis, I., Kögeler, J., Lampadariou, N., Pearson, T.H., Provost, P., Thetmeyer, H., Vögele, B., White, P., 2004. MERAMOD (version 1.4). Model for predicting the effects of Mediterranean fish farms. EU Project Q5RS-2000-31779.

Cromey, C.J., Nickell, T.D., Black, K.D., 2002. DEPOMOD-modelling the deposition and biological effects of waste solids from marine cage farms. Aquaculture 214, 211-239.

Dalsgaard, T., Krause-Jensen, D., 2006. Monitoring nutrient release from fish farms with macroalgal and phytoplankton bioassays. Aquaculture 256, 302-310.

Delgado, O., Grau, A., Pou, S., Riera, F., Massuti, C., Zabala, M., Ballesteros, E., 1997. Seagrass regression caused by fish cultures in Fornells Bay (Menorca, Western Mediterranean). Oceanologica Acta 20, 557–563.

Delgado, O., Ruiz, J..M., Pérez, M., Romero, J., Ballesteros, E., 1999. Effects of fish farming on seagrass (*Posidonia oceanica*) in a Mediterranean bay: seagrass decline after organic loading cessation. Oceanologica Acta 22, 109-117.

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

Deutsch, B., Voss, M., 2006. Anthropogenic nitrogen input traced by means of $\delta^{15}N$ values in macroalgae: Results from in-situ incubation experiments. Science of the Total Environment 366, 799-808.

Dimech, M., Borg, J.A., Schembri, P.J., 2000. Structural changes in a *Posidonia oceanica* meadow exposed to a pollution gradient from a marine fish farm in Malta (Central Mediterranean). Biología Marina Mediterránea 7, 361-364.

Dolenec, T., Lojen, S., Kniewald, G., Dolenec, M., Rogan., N., 2007. Nitrogen stable isotope composition as a tracer of fish farming in invertebrates *Aplysina aerophoba, Balanus perforatus and Anemonia sulcata* in central Adriatic. Aquaculture 262, 237-249.

Dosdat, A., Gaumet, F., Chartois, H., 1995. Marine aquaculture effluent monitoring-methodological approach to the evaluation of nitrogen and phosphorus excretion by fish. Aquacultural Engineering 14, 59–84.

Duarte, C.M., 1990. Seagrass nutrient content. Marine Ecology Progress Series 67, 201-207.

Duarte, C.M., 1995. Submerged aquatic vegetation in relation to different nutrient regimes. Ophélia 41, 87-112.

Dudley, B.D., 2007. Quantitative ecological impact assessments using natural abundance carbon and nitrogen stable isotope signatures. Thesis: Victoria University of Wellington.

Dudley, R.W., Panchang, V.G., Newell, C.R., 2000. Application of a comprehensive modeling strategy for the management of net-pen aquaculture waste transport. Aquaculture 187, 319-349.

Erskine, P.D., Bergstrom, D.M., Schmidt, S., Stewart, G.R., Tweedie, C.E., Shaw, J.D., 1998. Subantarctic Macquarie Island - a model ecosystem for studying animal-derived nitrogen sources using ^{15}N natural abundance. Oecologia 117, 187-193.

Evrard, V., Kiswara, W., Bouma, T.J., Middelburg, J.J., 2005. Nutrient dynamics of seagrass ecosystems: ^{15}N evidence for the importance of particulate organic matter and root Systems. Marine Ecology Progress Series 295, 49-55.

FAO, 2006. The state of world fisheries and aquaculture. Fisheries and Aquaculture Department. Food and Agriculture Organization of the United Nations, Rome, Italy.

FAO, 2007. The State of World Fisheries and Aquaculture. FAO Fisheries and Aquaculture Department. Food and Agriculture Organization of the United Nations, Rome, 2007.

FAO, 2008. The State of World Fisheries and Aquaculture (SOFIA) 2008. FAO. Fisheries department. Roma. 2009.

FAO/FIDI. 2006. "Aquaculture production, quantity 1950-2004" and "Aquaculture production, value 1984-2004". *FISHSTAT Plus* - Universal software for fishery statistical time series [online or CDROM]. Food and Agriculture Organization of the United Nations. Available at: http://www.fao.

FAO/GFCM/ICCAT. 2005. Report of the third meeting of the Ad Hoc GFCM/ICCAT Working Group on Sustainable Bluefin Tuna Farming/ Fattening Practices in the Mediterranean. Rome, 16–18 March 2005. FAO Fisheries Report. No. 779. Rome, FAO. 108 pp.

Feldmann, J., 1937. Recherches sur la végétation marine de la Méditerranée. La côte des Albères. Rev. Algol. 10, 73-254.

Felsing M., Glencross B., Telfer T., 2005. Preliminary study on the effects of exclusion of wild fauna from aquaculture cages in a shallow marine environment. Aquaculture 243, 159-174.

Fernández-Jover, D. Sanchez-Jerez, P., Bayle-Sempere, J., Carratala, A., Leon, V.M., 2007. Addition of dissolved nitrogen and dissolved organic carbon from wild fish faeces and

food around Mediterranean fish farms: implications for waste-dispersal models. Journal of Experimental Marine Biology and Ecology 340, 160-168.

Fong, P., Boyer, K.E., Kamer, K., Boyle, K.A., 2003. Influence of initial tissue nutrient status of tropical marine algae on response to nitrogen and phosphorus additions. Marine Ecology Progress Series 262, 111-123.

Fong, P., Boyer, K.E., Zedler, J.B., 1998. Developing an indicator of nutrient enrichment in coastal estuaries and lagoons using tissue nitrogen content of the opportunistic alga, *Enteromorpha intestinalis* (L Link). Journal of Experimental Marine Biology and Ecology 231, 63-79.

Fong, P., Fong, J.J., Fong, C.R., 2004. Growth, nutrient storage, and release of dissolved organic nitrogen by *Enteromorpha intestinalis* in response to pulses of nitrogen and phosphorus. Aquatic Botany 78, 83-95.

Fong, P., Kamer, K., Boyer, K.E., Boyle, K.A., 2001. Nutrient content of macroalgae with differing morphologies may indicate sources of nutrients to tropical marine systems. Marine Ecology Progress Series 220, 137-152.

Fourqurean, J.W., Moore, T.O., Fry, B., Hollibaugh, J.T., 1997. Spatial and temporal variation in C:N:P ratios, $\delta^{15}N$ and $\delta^{13}C$ of eelgrass *Zostera marina* as indicators of ecosystem processes, Tomales Bay, California, USA. Marine Ecology Progress Series 157, 147–157.

Fourqurean, J.W., Zieman, J.C., Powell, G.V.N., 1992. Phosphorus limitation of primary production in Florida Bay: Evidence from the C:N:P ratios of the dominant seagrass *Thalassia testudinum*. Limnology Oceanography 37, 162-171.

Frankovich, T.A., Fourqurean, J.W., 1997. Seagrass epiphyte loads along a nutrient availability gradient, Florida Bay, USA. Marine Ecology Progress Series 159, 37-50.

Franson, M.A.H., 1985. Method 424-F. In: Standard Methods for the Examination of Water and Wastewater, 16th ed. APHA, AAWA, and WPCF, Washington, DC, pp. 448-450.

Frederiksen, M.S., Holmer, M., Pérez, M., Invers, O., Ruiz, J.M., Knudsen B.B., 2008. Effect of increased sediment sulphide concentrations on the composition of stable sulphur

isotopes ($\delta^{34}S$) and sulphur accumulation in the seagrasses Zostera marina and Posidonia oceanica. Journal of Experimental Marine Biology and Ecology 358, 98-109.

Fry, B. 2006. Stable isotope ecology, New York, NY : Springer, 308 p.

García-Sanz, M., Ruiz, J.M., Ruiz, M., Pérez, M., González, M.N., García, R., in press An evaluation of a macroalgal bioassay tool for assessing the spatial extent of nutrient release from offshore fish farms. Marine Environmental Research.

Gartner, A., Lavery, P., Smit, A.J., 2002. Use of $\delta^{15}N$ signatures of different functional forms of macroalgae and filter-feeders to reveal temporal and spatial patterns in sewage disposal. Marine Ecology Progress Series 235, 63-73.

GESAMP (IMO /FAO /UNESCO -IOC /UNIDO /WMO /IAEA /UN /UNE P Joint Group of Experts on Scientific Aspects of Marine Environmental Protection) 2008. Assessment and communication of environmental risks in coastal aquaculture. Rome, FAO. *Reports and Studies GESAMP* No. 76: 198 pp.

GESAMP, 1996. Monitoring the ecological effects of coastal aquaculture wastes. GESAMP, Rome, Rep. Stud., nº 57, 38 p.

Graham, J.B., Dickson, K.A., 2001. Anatomical and physiological specializations for endothermy. In: Tuna: Physiology, Ecology and Evolution (Block, B.A. & Stevens, E.D. eds), pp. 121–166. Academic Press, San Diego.

Green, E.P. and Short, F.T. (eds.). 2003. World Atlas of Seagrasses. University of California Press, Los Angeles, 298 pp.

Grice, A.M., Loneragan, N.R., Dennison, W.C., 1996. Light intensity and the interactions between physiology, morphology and stable isotope ratios in five species of seagrass. Journal of Experimental Marine Biology and Ecology 195, 91-110.

Gunn, J., Block, B., 2001. Advances in acoustic, archival and satellite tagging of tunas. In: Tuna: Physiology, Ecology and Evolution (Block, B.A. & Stevens, E.D. eds), pp. 167–224. Academic Press, San Diego, CA.

Guo, L., Li, Z., 2003. Effects of nitrogen and phosphorus from fish cage-culture on the communities of a shallow lake in middle Yangtze River basin of China. Aquaculture 226, 201-212.

Hall, P.O.J., Holby, O., Kollberg, S., Samuelsson, M.O., 1992. Chemical fluxes and mass balances in a marine fish cage farm: IV. Nitrogen. Mar. Ecol. Prog. Ser. 89, 81-91.

Halpern, B.S., Walbridge, S., Selkoe, K.A., Kappel, C.V., Micheli, F., D'Agrosa, C., Bruno, J.F., Casey, K.S., Ebert, C., Fox, H.E., Fujita, R., Heinemann, D., Lenihan, H.S., Madin, E.M.P., Perry, M.T., Selig, E.R., Spalding, M., Steneck, R., Watson, R., 2008. A Global Map of Human Impact on Marine Ecosystems. Science 319, 948-952.

Halwart, M., Soto, D., Arthur, J.R., (eds.), 2007. Cage Aquaculture – Regional reviews and global overview. FAO Fisheries Technical Paper nº 498. Roma, 255p.

Heaton, T.H.E., 1986. Isotopic studies of nitrogen pollution in the hydrosphere and atmosphere: a review. Chemical Geology 59, 87-102.

Heck, K.L., Pennock, J.R., Valentine, J.F., Coen, L.D., Sklenar, S.A., 2000. Effects of nutrient enrichment and small predators density on seagrass ecosystems: an experimental assessment. Limnology and Oceanography 45, 1041-1057.

Heikoop, J.M., Dunn, J.J., Risk, M.J., Sandeman, I.M., Schwarcz, H.P., Waltho, N., 1998. Relationship between light and the $\delta^{15}N$ of coral tissue: Examples from Jamaica and Zanzibar. Limnology and Oceanography 43, 909-920.

Hemminga, M.A., Duarte, C.M., 2000. Seagrass ecology. Cambridge University Press.

Hoch, M.P., Fogel, M.L., Kirchman, D.L., 1992. Isotope fractionation associated with ammonium uptake by a marine bacterium. Limnology and Oceanography 37, 1447-59.

Hoffmann, L., Clarisse, S., Detienne, X., Goffart, A., Renard, R., Demoulin, V., 1988. Evolution of the populations of *Cystoseira balearica* (Phaeophyceae) and epiphytic Bangiophyceae in the Bay of Calvi (Corsica) in the last eight years. Bulletin de la Société Royale de Liège 4-5, 263-273.

Holby, O., Hall, P.O.J., 1991. Chemical fluxes and mass balances in a marine fish cage farm. II. Phosphorus. Mar. Ecol. Prog. Ser. 70, 263-272.

Holmer, M., Argyrou, M., Dalsgaard, T., Danovaro, R., Díaz-Almela, E., Duarte, C.M., Frederiksen, M., Karakassis, I., Marbà, N., Mirto, S., Pérez, M., Pusceddu, A., Tsapkasis, M., in press. Effects of fish farm waste on *Posidonia oceanica* meadows: synthesis and provision of monitoring and management tools. Marine Pollution Bulletin

56, 1618-1629.

Holmer, M., Frederiksen, M.S., 2007. Stimulation of sulfate reduction rates in Mediterranean fish farm sediments inhabited by the seagrass *Posidonia oceanica*. Biogeochemistry 85, 169-184.

Holmer, M., Kristensen, E., 1992. Impact of fish cage farming on metabolism and sulfate reduction of underlying sediments. Marine Ecology Progress Series 80, 191-201.

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

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

Holmer, M., Wildish, D., Hargrave, B., 2005. Organic enrichment from marine finfish aquaculture and effects on sediment biogeochemical processes. Handbook of Environmental Chemistry 5, 181-206.

Horrocks, J., Stewart, G.R., Dennison, W.C., 1995. Tissue nutrient content of *Gracilaria spp.* (Rhodophyta) and water quality along an estuarine gradient. Marine and Freshwater Research 46, 975-983.

Invers, O., Kraemer, G.P., Pérez, M., Romero, J., 2004. Effects of nitrogen addition on nitrogen metabolism and carbon reserves in the temperate seagrass *Posidonia oceanica*. Journal of Experimental Marine Biology and Ecology 303, 97-114.

Invers, O., Ruiz, J.M., Pérez, M., 2003. Fish farm effects on *Posidonia oceanica* community structure along transects from cages (Cyprus and Sicily) Annex 4.2 MEDVEG project Annual Report. www.medveg.dk.

Islam, M.S., 2005. Nitrogen and Phosphorus budget in coastal and marine cage aquaculture and impacts of effluent loading on ecosystem: review and analysis towards model development. Marine Pollution Bulletin 50, 48-61.

Jones, A.B., O'Donohue, M.J., Udy, J., Dennison, W.C., 2001. Assessing ecological impacts of shrimp and sewage effluent: biological indicators with standard water quality analyses. Estuarine, Coastal and Shelf Science 52, 91-109.

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

Kamer, K., Boyle, K.A., Fong, P., 2001. Macroalgal bloom dynamics in a highly eutrophic southern California estuary. Estuaries 24, 623-35.

Kamer, K., Fong, P., 2001. Nitrogen enrichment ameliorates the negative effects of reduced salinity in the green macroalga *Enteromorpha intestinalis*. Marine Ecology Progress Series 218, 87-93.

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

Karakassis I., Hatziyanni E., 2000. Benthic disturbance due to fish farming analyzed under different levels of taxonomic resolution. Marine Ecology Progress Series 203, 247-253.

Karakassis I.,1998. Aquaculture and coastal marine biodiversity. Océanis, 24:271-286

Karakassis, I., 2001. Ecological effects of fish farming in the Mediterranean. Cahiers Options Méditerranéenes 55, 15-22.

Karakassis, I., Pitta, P.,Krom, M.D., 2005. Contribution of fish farming to the nutrient loading of the Mediterranean. Scientia Marina 68, 313-321.

Karakassis, I., Tsapakis, M., Astillan, E., Pitta, P., 2001. Diel variation of nutrients and chlorophyll in the sea bream and sea bass cages in the Mediterranean. Fresenius Environmental Bulletin 10, 278-283.

Karakassis, I., Tsapakis, M., Hatziyanni, E., 1998. Seasonal variability in sediment profiles beneath fish farm cages in the Mediterranean. Marine Ecology Progress Series 162, 243-252.

Kaushal, S.S., Lewis, W.M., McCutchan. J.H., 2006. Land use change and nitrogen enrichment of a Rocky Mountain watershed. Ecological Applications 16, 299-312.

Kohl, J.G., Woitke, P., Kühl, H., Dewender, M., König, G., 1998. Seasonal changes in dissolved amino acids and sugars in basal culm internodes as physiological indicators of the C/N-balance of *Phragmites australis* at littoral sites of different trophic status. Aquatic Botany 60, 221-240.

Korsmeyer, K.E., Dewar, H., 2001. Tuna metabolism and energetics. In: Tuna: Physiology, Ecology and Evolution (Block, B.A. & Stevens, E.D. eds), pp. 36–78. Academic Press, San Diego.

La Rosa, T., Mirto, S., Favaloro. E., Savona, B., Sarà, G., Danovaro, R., Mazzola, A. 2002. Impact on the water column biogeochemistry of a Mediterranean mussel and fish farm. Water Research 36, 713-721.

La Rosa, T., Mirto, S., Mazzola, A., Danovaro, R., 2001. Differential responses of benthic microbes and meiofauna to fish-farm disturbance in coastal sediments. Environmental Pollution 112, 427-434.

Larkum, W.D., Orth, R.J., Duarte, C.M. (eds.). 2006. Seagrasses: Biology, Ecology and Conservation. Springer, Dordrecht, 691 pp.

Lathja, K., Michener, R.H., 1994. Stable isotopes in ecology and environmental science. Blackwell Scientific Publications, Oxford.

Lee, K.S. Dunton, K.H., 1999. Influence of sediment nitrogen-availability on carbon and nitrogen dynamics in the seagrass *Thalassia testudinum*. Marine Biology 134, 217-226.

Lee, K.S., Short, F.T., Burdick, D.M., 2004. Development of a nutrient pollution indicator using the seagrass*, Zostera marina*, along nutrient gradients in three New England estuaries. Aquatic botany 78, 197-216.

Lefebvre, S., Bacher, C., Meuret, A., Hussenot, J., 2001. Modelling nitrogen cycling in a mariculture ecosystem as a tool to evaluate its outflow. Estuarine, Coastal and Shelf Science 52, 305-325.

Lemarie. G., Martin J.L.M., Dutto G., Garidou C., 1998. Nitrogen and phosphorus waste production in a owthrough land-based farm of European seabass (*Dicentrarchus labrax*). Aquatic Living Resources 11, 247-254.

Leoni, V., Pasqualini, V., Pergent-Martini, C., Vela, A., Pergent, G., 2006. Morphological responses of *Posidonia oceanica* to experimental nutrient enrichment of the canopy water. Journal of Experimental Marine Biology and Ecology 339, 1-14.

Leoni, V., Pasqualini, V., Pergent-Martini, C., Vela, A., Pergent, G., 2007. Physiological responses of *Posidonia oceanica* to experimentalnutrient enrichment of the canopy water. Journal of Experimental Marine Biology and Ecology 349, 73-83.

Lepoint, G., Dauby, P., Fontaine, M., Bouquegneau J.M., Gobert, S., 2003. Carbon and Nitrogen Isotopic Ratios of the Seagrass *Posidonia oceanica*: Depth-related Variations. Botanica Marina 46, 555-561.

Lepoint, G., Dauby, P., Gobert, S., 2004. Applications of C and N stable isotopes to ecological and environmental studies in seagrass ecosystems. Marine Pollution Bulletin 49, 887-891.

Lepoint, G., Frédérich, B., Gobert, S, Parmentier, E., 2008. Isotopic ratios and elemental contents as indicators of seagrass C processing and sewage influence in a tropical macrotidal ecosystem (Madagascar, Mozambique Channel). Scientia Marina 72, 109-117.

Lepoint, G., Jacquemart, J., Bouquegneau, J.M., Demoulin, V., Gobert, S, 2007. Field measurements of inorganic nitrogen uptake by epiflora components of the seagrass *Posidonia oceanica* (Monocotyledons, Posidoniaceae). Journal of Phycology 43, 208-218.

Liao, C.T., Lin, C.H., 2001. Physiological adaptation of crop plants to flooding stress. Proceedings of the National Science Council 25, 148-157.

Lin, D.T., Fong, P., 2008. Macroalgal bioindicators (growth, tissue N, $\delta^{15}N$) detect nutrient enrichment from shrimp farm effluent entering Opunohu Bay, Moorea, French Polynesia. Marine Pollution Bulletin 56, 245-249.

Lin, H.J., Nixon, S.W., Taylor, D.I., Granger, S.L., Buckley B.A, 1996. Responses of epiphytes on eelgrass, *Zostera marina* L., to separate and combined nitrogen and phosphorus enrichment. Aquatic Botany 52, 243-258.

Lobban, C.S., Harrison, P.J., 1997. Seaweed Ecology and Physiology. Cambridge University Press, Cambridge.

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

Lupatsch I., Kissil, G.W., 1998. Predicting aquaculture waste from gilthead seabream (*Sparus aurata*) culture using nutritional approach. Aquatic Living Resources 11, 265-268.

Lupatsch, I., Katz, T., Angel, D.L., 2003. Removal of fish farm effluents by grey mullets: a nutritional approach. Aquatic Living Resources 34, 1367-1377.

Lyngby, J.E. 1990. Monitoring of nutrient availability and limitation using the marine macroalgae, *Ceramium rubrum* (Huds.) C. Ag. Aquatic Botany 38, 153-161.

Lyngby, J.E., Mortensen, S., Ahrensberg, N., 1999. Bioassessment techniques for monitoring of eutrophication and nutrient limitation in coastal ecosystems. Marine Pollution Bulletin 39, 212-223.

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

Machias, A., Karakassis, I., Somarakis, S., Giannoulaki, M., Papadopoulou, K.N., Smith, C., 2005. The response of demersal fish communities to the presence of fish farms. Marine Ecology Progress Series 288, 241–250.

Macko, S.A., Ostrom, N.E., 1994. Pollution Studies Using Stable Isotopes, Chapter 3. In 'Stable Isotopes in Ecology and Environmental Science'. (Eds Lajtha, K. and Michener, R.H.) pp. 45-62. (Blackwell Scientific Publications: Oxford).

Maldonado, M., Carmona, M.C., Echeverría, Y., Riesgo, A., 2005. The environmental impact of Mediterranean cage fish farms at semi-exposed locations: does it need a re-assessment?. Helgoland Marine Research 59, 121-135.

Mann, K.H., 1982. Ecology of coastal waters: a systems approach, vol. 8 (eds. D. J. Anderson, P. Greig-Smith and F. A. Pitelka). London: Blackwell Scientific Publications.

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

Marbà, N., Santiago, R., Díaz-Almela, E., Álvarez, E., Duarte,C.M., 2006. Seagrass (*Posidonia oceanica*) vertical growth as an early indicador of fish farm-derived stress. Estuaine Coastal and Shelf Science 67, 475-483.

Marbà, N., Terrados, J., 2004. Las praderas de *Cymodocea nodosa*. Distribución y requerimientos ecológicos: 133-134, en Luque, A.A. y Templado, J. (Coords.) Praderas y bosques marinos de Andalucía, Consejería de Medio Ambiente, Junta de Andalucía, Sevilla.

Margalef, R. (ed.) 1989. Western Mediterranean. London, Pergamon Press.

Mariotti, A., 1983. Atmospheric nitrogen as a reliable standard for natural ^{15}N abundance measurements. Nature, 685–687.

Mariotti, A., Mariotti, F., Champigny, M.-L., Amarger, N., Moyse, A., 1982. Nitrogen isotope fractionation associated with nitrate reductase activity and uptake of $NO3^-$ by pearl millet. Plant Physiology 69, 880-4.

Martínez-Crego, B., A. Vergés, T. Alcoverro and J. Romero. 2008. Selection of multiple seagrass indicators for environmental biomonitoring. Marine Ecology Progress Series 361, 93-109.

Mazzola, A., Mirto, S., La Rosa, T., Fabiano, M., Danovaro, R., 2000. Fish farming effects on benthic community structure in coastal sediments: analysis of meiofaunal recovery. Journal of Marine Science 57, 1454-1461.

McClelland, J.W., Valiela, I., 1998. Linking nitrogen in estuarine producers to land-derived sources. Limnology and Oceanography 43, 577-85.

McGhie, T.K., Crawford, C.M., Mitchell, I.M., O'Brien, D., 2000. The degradation of fish-cage waste in sediments during fallowing. Aquaculture 187, 351–366.

McGlathery, K.J., 1995. Nutrient and grazing influences on a subtropical seagrass community. Marine Ecology Progress Series 122, 239-252.

McKee, K.L., Feller, I.C., Popp, M., Wanek, W., 2002. Mangrove isotopic (delta N-15 and delta C-13) fractionation across a nitrogen vs. phosphorus limitation gradient. Ecology 83, 1065-1075.

McKinney, R.A., Nelson, W.G., Charpentier, M.A., Wigand, C., 2001. Ribbed mussel nitrogen isotope signatures reflect nitrogen sources in coastal salt marshes. Ecological Applications 11, 203-14.

Merino, G.E., Piedrahita, R.H., Conklin, D.E., 2007. Ammonia and urea excretion rates of California halibut (*Paralichthys californicus*, Ayres) under farm-like conditions. Aquaculture 271: 227-243.

Midlen, A., Redding, T. 1998. Environmental Management for Aquaculture. (Series Ed. M.G. Poxton) Chapman and Hall Aquaculture Series 2, 240 pp.

Mirto, S., La Rosa, T., Gambi, C., Danovaro, R., Mazzola, A., 2002. Nematode community response to fish-farm impact in the western Mediterranean. Environmental Pollution 116, 203–214.

Moore, K.A., Wetzel, R.L., 2000. Seasonal variations in eelgrass (*Zostera marina* L.) responses to nutrient enrichment and reduced light availability in experimental ecosystems. Journal of Experimental Marine Biology and Ecology 244, 1-28.

Naldi, M., Wheeler, P.A., 1999. Changes in nitrogen pools in *Ulva fenestrate* (Chlorophyta) and *Gracilaria pacifica* (Rhodophyta) under nitrate and ammonium enrichment. Journal of Phycology 35, 70-77.

Neckles, T.A., Wetzel, R.L., Orth, R.J., 1993. Relative effects of nutrient enrichment and grazing on epiphyte-macrophyte (*Zostera marina L.*) dynamics. Oecologia 93, 285-295.

Needoba, J.A., Harrison. P.J., 2004. Influence of low light and a light: Dark cycle on NO_3^- uptake, intracellular NO_3^-, and nitrogen isotope fractionation by marine phytoplankton. Journal of Phycology 40, 505-516.

Needoba, J.A., Sigman, D.M., Harrison, P.J., 2004. The mechanism of isotope fractionation during algal nitrate assimilation as illuminated by the $^{15}N/^{14}N$ of intracellular nitrate. Journal of Phycology 40, 517-522.

Neori, A., Chopin, T., Troell, M., Buschmann, A.H., Kraemer, G.P., Halling, C., Shpigel, M., Yarish, C., 2004. Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. Aquaculture 231, 361-391.

Nordvarg, L., Johansson, T., 2002. The effects of fish farm effluents on the water quality in the Aland archipielago, Baltic Sea. Aquacultural Engineering 25, 253-279.

Panayotidis, P., Montesanto, B., Orfanidis, S., 2004. Use of low-budget monitoring of macroalgae to implement the European Water Framework Directive. Journal of Applied Phycology 16, 49–59.

Pantoja, S., Repeta, D.J., Sachs, J.P., Sigman., D.M., 2002. Stable isotope constrains on the nitrogen cycle of the Mediterranean Sea water column. Deep Sea Research Part I: Oceanography Research Pap. 49: 1609-1621.

Pasqualini V., Pergent-Martini C., Clabaut P., Pergent G., 1998. Mapping of *Posidonia oceanica* using aerial photographs and side-scan sonar : Application of the island of Corsica (France). Estuarine Coastal Shelf Science, 47, 359-367.

Pauly, D., Christensen, V., 1995. Primary production required to sustain global fisheries. Nature 374, 255-257.

Pedersen, M.F., Borum, J., 1997. Nutrient control of estuarine macroalgae: growth strategy and the balance between nitrogen requirements and uptake. Marine Ecology Progress Series 161, 155-63.

Pedersen, M.F., Paling, E.I., Walker, D.I., 1997. Nitrogen uptake and allocation in the seagrass *Amphibolis antartica.* Aquatic botany 56, 105-117.

Pennock, J.R., Velinsky, D.J., Ludlam, J.M., Sharp, J.H., Fogel, M.L., 1996. Isotopic fractionation of ammonium and nitrate during uptake by *Skeletonema costatum*: Implications for $\delta^{15}N$ dynamics under bloom conditions. Limnology and Oceanography 41, 451-459.

Pérez, M., García, T., Ruiz, J.M., Invers, O., 2008. Physiological responses of the seagrass *Posidonia oceanica* as indicators of fish farm impact. Marine Pollution Bulletin, 56, 869-879.

Pérez, M., Invers, O., Ruiz, J.M., Frederiksen, M.S., Holmer, M., 2007. Physiological responses of the seagrass *Posidonia oceanica* to elevated organic matter content in sediments: An experimental assessment. Journal of Experimental Marine Biology and Ecology 344, 149-160.

Pérez, M., Romero, J., Duarte, C.M., Sand-Jensen, K., 1991. Phosphorus limitation of *Cymodocea nodosa* growth. Marine biology 109, 129-133.

Pérez-Llorens, J.L, Niell, F.X., 1995. Short-term phosphate uptake kinetics in *Zostera noltii* Hornem: A comparison between excised leaves and sediment-rooted plants. Hydrobiologia 297, 17-27.

Pergent, G., Mendez, S., Pergent-Martini, C., Pasqualini, V., 1999. Preliminary data on the impact of fish farming facilities on *Posidonia oceanica* meadows in the Mediterranean. Oceanologica Acta 22, 95-107

Pergent-Martini, C., Boudouresque, C.F., Pasqualini, V., Pergent, G., 2006. Impact of fish farming facilities on *Posidonia oceanica* meadows: a review. Marine Ecology 27, 310-319.

Pergent-Martini, C., Pergent, G., Fernandez, C., Ferrat, L., 1999. Value and use of *Posidonia oceanica* as a biological indicator. In: Ozhan, E., (Ed), Land-ocean interactions: managing coastal ecosystems. Proceeding MEDCOAST 99-EMECS 99 Joint Conference MEDCOAST, Middle Esat Technical Univ. Publ, Ankara. 73-90.

Peterson, B.J., 1999. Stable isotopes as tracers of organic matter input and transfer in benthic food webs: a review. Acta Oecologica 20, 479-487.

Peterson, B.J., Frankovich, T.A., Zieman, J.C., 2007. Response of seagrass epiphyte loading to field manipulations of fertilization, gastropod grazing and leaf turnover rates. Journal of Experimental Marine Biology and Ecology 349, 61-72.

Peterson, B.J., Fry, B., 1987. Stable isotopes in ecosystem studies. Annual Review of Ecology, Evolution and Systematics 18, 293-320.

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

Pitta, P., Apostolaki, E.T., Giannoulaki, M., Karakassis, I., 2005. Mesoescale changes in the water column in response to fish farming zones in three coastal areas in the Eastern Mediterranean Sea. Estuarine, Coastal and Shelf Science 65, 501-512.

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

Pitta, P., Karakassis, I., Tsapakis, M., Zivanovic, S., 1999. Natural vs. Mariculture induced variability in nutrients and plankton in the Eastern Mediterranean. Hydrobiologia 391, 181-194.

Prado, P., Alcoverro, T., Martínez-Crego, B., Vergés, A., Pérez, M., Romero, J., 2007. Macrograzers strongly influence patterns of epiphytic assemblanges in seagrass meadows. Journal of Experimental Marine Biology and Ecology 350, 130-143.

Pregnall, A.M., Smith, R.D., Kursar, T.A., Alberte, R.S., 1984. Metabolic adaptation of *Zostera marina* (eelgrass) to diurnal periods of root anoxia. Marine Biology 83, 141-147.

Primavera, J.H., 2006. Overcoming the impacts of aquaculture on the coastal zone. Ocean and Coastal Management 49, 531-545.

Quinn, G.P., Keough, M.J., 2002. Experimental design and data analysis for biologists, Cambridge University Press.

Reyes, J., Sanson, M., Afonsocarrillo, J., 1995. Distribution and Reproductive Phenology of the Seagrass *Cymodocea nodosa* (Ucria) Ascherson in the Canary Islands. Aquatic Botany 50, 171-180.

Romero, J., 1989. Seasonal pattern of *Posidonia oceanica* production: Growth, age and renewal of leaves. In: Boudouresque CF, Meinesz A, FresiEand GravezV (eds) InternationalWorkshop on *Posidonia* Beds, pp 63–68. GIS Posidonie 2, Marseille.

Romero, J., Lee, K., Pérez, M., Mateo, M.A., Alcoverro, T., 2006. Nutrient Dynamics in Seagrass Ecosystems. In Larkum, A.W.D., Orth, R.J., Duarte, C.M. (eds) Seagrasses: biology, ecology, and conservation (Springer), pp. 227–254.

Romero, J., Martínez-Crego, B., Alcoverro, T., Pérez, M., 2007. A multivariate index based on seagrass *Posidonia oceanica* (POMI) to assess ecological status of coastal waters under the water framework directive (WFD). Marine Pollution Bulletin 55, 196-204.

Roque d 'Orbcastel, E., Blancheton, J.P., 2006. The wastes from marine fish production systems: characterization, minimization, treatment and valorization. World Aquaculture 37, 28–35.

Roque d'Orbcastel, E., Blancheton, J.P., Boujard, T., Aubin, J., Moutounet, Y., Przybyla, C., Belaud, A., 2008. Comparison of two methods for evaluating waste of a flow through trout farm. Aquaculture 274, 72-79.

Ruiz, J.M., Marco-Méndez, C., Sánchez-Lizaso, J.L., in press. Remote influence of off-shore fish farm waste on Mediterranean seagrass (*Posidonia oceanica*) meadows. Marine Environmental Research.

Ruiz, J.M., Marco-Méndez, C., Sánchez-Lizaso, J.L., in press. Remote influence of off-shore fish farm waste on Mediterranean seagrass (*Posidonia oceanica*) meadows. Marine Environmental Research.

Ruiz, J.M., Pérez, M., Romero, J., 2001. Effects of fish farm loadings on seagrass (*Posidonia oceanica*) distribution, growth and photosynthesis. Marine Pollution Bulletin 42,749-760.

Ruiz, J.M., Romero, J., 2001. Effects of *in situ* experimental shading on the Mediterranean seagrass *Posidonia oceanica.*Marine Ecology Progress Series 215, 107-120.

Sakami, T., Abo, K., Takayanagi, K., Toda, S., 2003. Effects of water mass exchange on bacterial communities in an aquaculture area during summer. Estuarine, Coastal and Shelf Science 56, 111-118.

Samocha, T.M., Lawrence, A.L., 1997. Shrimp farms' effluent waters, environmental impact and potential treatment methods. In Interactions between cultured species and naturally occurring species in the environment, ed. BJ Keller, pp. 35-8.

Sarà, G., 2007. Aquaculture effects on some physical and chemical properties of the water column: A meta-analysis. Chemistry and Ecology 23, 251-262.

Sarà, G., Scilipoti, D., Mazzola, A., Modica, A., 2004. Effects of fish farming waste to sedimentary and particulate organic matter in a southern Mediterranean area (Gulf of Castellammare, Sicily): a multiple stable isotope study ($\delta^{13}C$ and $\delta^{15}N$). Aquaculture 234, 199-213.

Sarà, G., Scilipoti, D., Milazzo, M., Modica, A., 2006. Use of stable isotopes to investigate dispersal of waste from fish farms as a function of hydrodynamics. Marine Ecology Progress Series 313, 261-270.

Savage C, Elmgren R., 2004. Macroalgal (*Fucus vesiculosus*) $\delta^{15}N$ values trace decrease in sewage influence. Ecological Applications 14, 517-26.

Schneider, O., Amirkolaie, A.K., Vera-Cartas, J., Eding, E.H., Schrama J.W., Verreth J.A.J., 2004. Digestibility, faeces recovery, and related C, N, and P balances of five feed ingredients evaluated as fishmeal alternatives in *Oreochromis niloticus* L. Aquaculture Research 35, 1370-1379.

Schneider, O., Sereti, V., Eding, E.H., Verreth, J.A.J., 2005. Analysis of nutrient flows in integrated intensive aquaculture systems. Aquacultural Engineering 32, 379-40.

SGPM. Secretaría General de Pesca Marítima (2001) "Libro blanco de la acuicultura en España". Centro de Publicaciones del Ministerio de Agricultura, Pesca y Alimentación. Tomos I y II: 521 p.

Short, F.T., Burdick, D.M., 1996. Quantifying eelgrass habitat loss in relation to housing development and nitrogen loading in Waquoit Bay, Massachusetts. Estuaries 19, 730-739.

Short, F.T., Burdick, D.M., Kaldy, J.E., 1995. Mesocosm experiments quantify the effects of eutrophication on eelgrass, *Zostera marina*. Limnology Oceanography 40, 740-749.

Short, F.T., Wyllie-Echeverria, S., 1996. A Review of Natural and Human-induced Disturbance of Seagrasses. Environmental Conservation 23, 17-27.

Simboura, N., Zenetos, A., 2002. Benthic indicators to use in ecological quality classification of Mediterranean soft bottom marine ecosystems, including a new biotic index. Mediterranean Marine Science 3, 77-111.

Smith, R.D., Pregnall, A.M., Alberte, R.S., 1988. Effects of anaerobiosis on root metabolism of *Zostera marina* (eelgrass): implications for survival in reducing sediments. Marine Biology 98, 131-141.

Solorzano, 1969. Determination of ammonia in natural waters by phenol hypochlorite method, Limnology and Oceanography 14, 799-801.

Soltan, D., Verlaque, M., Boudouresque, C.F., Francour, P., 2001. Changes in macroalgal communities in the vicinity of the Mediterranean sewage outfall after the setting up of a treatment plant. Marine Pollution Bulletin 42, 59–70.

Soto, D., Norambuena, F., 2004. Evaluation of salmon farming effects on marine systems in the inner seas of southern Chile: a large-scale mensurative experiment. Journal of Applied Ichthyology 20, 493-501.

Spackman, D.H., Stein, W.H., Moore, S., 1958. Automatic recording apparatus for use in the chromatography of amino acids. Analytical Chemistry 30, 1190-1206.

Stergiou, K.I., Tsikliras, A.C., Pauly, D., 2009. Farming up the Mediterranean food webs. Conservation Biology 23, 230-232.

Subasinghe, R. (2004). Aquaculture in the global food supply. Rome: FAO.

Tacon, A.G.J., Halwart, M., 2007. Cage aquaculture: a global overview. In M. Halwart, D. Soto and J.R. Arthur (eds). Cage aquaculture – Regional reviews and global overview, pp. 1–16. FAO Fisheries Technical Paper. No. 498. Rome, 241 pp.

Tewfik, A., Rasmussen, J.B., McCann, K.S., 2005. Anthropogenic enrichment alters a marine benthic food web. Ecology 86, 2726-2736.

Tomasko, D.A., Lapointe, B.E., 1991. Productivity and biomass of *Thalassia testudinum* as related to water column nutrient availability and epiphyte levels: field observations and experimental studies. Marine Ecology Progress Series 75, 9-17.

Touchette, B.W., Burkholder, J.M., 2000. Review of nitrogen and phosphorus metabolism in seagrasses. Journal of Experimental Marine Biology and Ecology 250, 133-167.

Touchette, B.W., Burkholder, J.M., Glasgow, H.B., 2003. Variations in eelgrass (*Zostera marina* L.) morphology and internal nutrient composition as influenced by increased temperature and water column nitrate. Estuaries 26, 142-155.

Tsapakis, M., Pitta, P., Karakassis, I., 2006. Nutrients and fine particulate matter released from sea bass (*Dicentrarchus labrax*) farming. Aquatic Living Resources 19, 69-75

Udy, J.W., Dennison W.C., 1997. Physiological responses of seagrasses used to identify anthropogenic nutrient inputs. Marine and Freshwater Research 47, 605-614.

Umezawa, Y., Miyajima, T., Yamamuro, M., Kayanne, H., Koike, I., 2002. Fine-scale mapping of land-derived nitrogen in coral reefs by $\delta^{15}N$ in macroalgae. Limnology and Oceanography 47, 1405-1416.

Underwood, A.J., 1997. Experiments in ecology. Their Logical and Interpretation Using Analysis of Variance. Cambridge Univ. Press, p. 504.

UNEP, 2002. Vital Water Graphics. An Overview of the State of the World´s Fresh and Marine Waters. UNEP, Nairobi, Kenya. ISBN: 92-807-2236-0.

UNEP/GRID-Arendal, 2007. Coastal population and altered land cover in coastal zones (100 km of coastline). UNEP/GRID-Arendal Maps and Graphics Library. http://maps.grida.no/go/graphic/coastal-population-and-altered-land-cover-in-coastal-zones-100-km-of-coastline.

Vezzulli, L., Chelossi, E., Riccardi, G., Fabiano, M., 2002. Bacterial community structure and activity in fish farm sediments on the Ligurian sea (Western Mediterranean). Aquaculture International 10, 123-141.

Vezzulli, L., Moreno, M., Marin, V., Pezzati, E., Bartoli, M., Fabiano, M., 2008. Organic waste impact of capture-based Atlantic bluefin tuna aquaculture at an exposed site in the Mediterranean Sea. Estuarine, Coastal and Shelf Science 78, 369-384.

Vita, R., Marin, A., Jimenez-Brinquis, B., Cesar, A., Marin-Guirao, L., Borredat, M., 2004. Aquaculture of Bluefin tuna in the Mediterranean: evaluation of organic particulate wastes. Aquaculture Research 35, 1384-1387.

Vizzini, S., Mazzola, A., 2004. Stable isotope evidence for the environmental impact of a land-based fish farm in the western Mediterranean. Marine pollution Bulletin 49, 61-70.

Vizzini, S., Mazzola, A., 2006. The effects of anthropogenic organic matter inputs on stable carbon and nitrogen isotopes in organisms from different trophic levels in a southern Mediterranean coastal area. Science of the Total Environment 368, 723-731.

Vizzini, S., Sarà, G., Mateo, M.A., Mazzola, A., 2003. $\delta^{13}C$ and $\delta^{15}N$ variability in *Posidonia oceanica* associated with seasonality and plant fraction. Aquatic Botany 76, 195-202.

Wada, E., Hattori, A., 1978. Nitrogen isotope effects in the assimilation of inorganic nitrogenous compounds by marine diatoms. Geomicrobiology 1, 85-101.

Wada, E., Minagawa, M., Mizutani, H., Tsujo, T., Imaizumi, R. & Karasawa, K. 1987. Biogeochemical studies on the transport of organic matter along the Otsuchi River Watershed, Japan. Estuarine and Coastal Shelf Science 25, 321-336.

Wallentinus, I. 1984. Comparisons of nutrient uptake rates for Baltic macroalgae with different thallus morphologies. Marine Biology 80, 215-225.

Waser, N.A.D., Harrison, P.J., Nielsen, B., Calvert, S.E., Turpin, D.H. 1998a. Nitrogen isotope fractionation during the uptake and assimilation of nitrate, nitrite, ammonium, and urea by a marine diatom. Limnology and Oceanography 43, 215–24.

Waser, N.A.D., Yin, K., Yu, Z., Tada, K., Harrison, P.J., Turpin, D.H., Calvert, S.E. 1998b. Nitrogen isotope fractionation during nitrate, ammonium and urea uptake by marine diatoms and coccolithophores under various conditions of N availability. Marine Ecology Progress Series 169, 29-41.

Wear, D.J., Sullivan, M.J., Moore, A.D., Millie, D.F., 1999. Effects of water-column enrichment on the production dynamics of three seagrass species and their epiphytic algae. Marine Ecology Progress Series 179, 201-213.

Wolanski, E., Spagnol, S., Thomas, S., Moore, K., Alongi, D.M., Trott, L., Davidson, A., 2000. Modelling and visualizing the fate of shrimp pond effluent in a mangrove-fringed tidal creek. Estuarine Coastal and Shelf Science 50, 85-97.

Wu R.S.S., 1995. The environmental impact of marine fish culture: Towards a sustainable future. Marine Pollution Bulletin 31, 159-166.

Wu, R.S.S., Lam, K. S., Mackay, D. W., Lau, T. C. and Yam, V., 1994. Impact of marine fish farming on water quality and bottom sediment: a case study of the sub-tropical environment. Marine Environmental Research 38, 115-145.

Yamamuro, M., Kayanne, H., Yamano, H., 2003. $\delta^{15}N$ of seagrass leaves for monitoring anthropogenic nutrient increases in coral ref. ecosystems. Marine Pollution Bulletin 46, 452-458.

Yamamuro, M., Umezaba, Y., Koike, I., 2004. Internal variations in nutrient concentrations and the C and N stable isotope ratios in leaves of the seagrass *Enhalus acoroides*. Aquatic Botany 79, 95-102.

Ye, L.X., Ritz, D.A., Fenton, G.E., Lewis, M.E., 1991. Tracing the influence on sediments of organic waste from a salmonid farm using stable isotope analysis. Journal of Experimental Marine Biology and Ecology 145, 161-174.

Yemm, E.W., Williams, A.J., 1954. The estimation of carbohydrates in plant extracts by anthrone. Biochemistry 57, 508-514.

Zar, J.H., 1999. Biostatistical Análisis. Prentice Hall, USA, 660 pp.

Zimmerman, R.C., Alberte, R.S., 1996. Effects of light/dark transition on carbon translocation in eelgrass *Zostera marina* seedlings. Marine Ecology Progress Series 136, 305-309.