Integration of physiological energetics, biometrics, proximate composition and fatty acids as biomarkers to assess the utilization of fish feed waste by mussels growing near fish cages

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## Doctoral Thesis
## 2015

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**CERTIFICAN:**

Que la presente Tesis Doctoral titulada: Integration of physiological energetics, biometrics, proximate composition and fatty acids as biomarkers to assess the utilization of fish feed waste by mussels growing near fish cages, que presenta JADE IRISARRI CAL, ha sido realizada bajo nuestra dirección en el Grupo de Ecofisiología, biomarcadores y gestión sostenible de Bivalvos (EsMaBa) del Instituto de Investigaciones Marinas (IIM-CSIC), y autorizamos su presentación ante el tribunal correspondiente bajo la modalidad de tesis por compendio de artículos de investigación, con el visto bueno del tutor, Gonzalo Méndez Martínez, profesor titular la Universidad de Vigo

Vigo, 27 de enero de 2015

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# Scientific publications in this thesis

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**2. Irisarri, J.,** Fernández-Reiriz, M.J., Cranford, P.J., Labarta, U., 2014. Effects of seasonal variations in phytoplankton on the bioenergetic responses of mussels (*Mytilus galloprovincialis*) held on a raft in the proximity of red sea bream (*Pagellus bogaraveo*) net-pens. Aquaculture 428-429, 41-53.

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**4. Irisarri, J.,** Fernández-Reiriz, M.J., De Troch, M., Labarta, U., 2014. Fatty acids as tracers of trophic interactions between seston, mussels and biodeposits in a coastal embayment of mussel rafts in the proximity of fish cages. Comparative Biochemistry and Physiology, Part B 172-173, 105-115.

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**6. Irisarri, J.,** Fernández-Reiriz, M.J., Labarta, U., 2015. Temporal and spatial variations in proximate composition and Condition Index of mussels *Mytilus galloprovincialis* cultured in suspension in a shellfish farm. Aquaculture 435, 207-216.

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# INDEX

# Resumen en Español

La acuicultura es el sector alimentario con más rápido crecimiento del mundo. Este crecimiento ha sido impulsado por el declive de la pesca y la creciente demanda de productos marinos de una población que prosigue expandiéndose. España es el segundo mayor productor de mejillón del mundo y uno de los principales países europeos que cultivan pescado. La región de Galicia produce 250,000 toneladas anuales de mejillón *Mytilus galloprovincialis* y esta cosecha representa el 99% de la producción total española. El cultivo del mejillón se realiza en bateas, donde el mejillón crece suspendido en cuerdas y se alimenta del seston, el conglomerado de partículas orgánicas –fitoplancton, bacterias, partículas fecales– e inorgánicas en suspensión en la columna de agua. A diferencia del cultivo de mejillón, la acuicultura de peces carnívoros como el salmón o el besugo, es intensiva, es decir, necesita un aporte exógeno de alimento. Uno de los mayores problemas asociados al cultivo de peces carnívoros en jaulas es la producción de gran cantidad de desechos orgánicos. Estos residuos de pescado pueden ser o bien desechos particulados –partículas de pienso no consumidas y heces– o bien productos resultantes del metabolismo que se disuelven en el agua. Los residuos derivados del cultivo de peces pueden aumentar el contenido orgánico de los sedimentos, afectar a las comunidades bentónicas, causar eutrofización del agua o incluso inducir proliferaciones de fitoplancton en las cercanías de las jaulas de peces. En los últimos años, los mejillones se han propuesto como candidatos para biomitigar el impacto ambiental derivado de los desechos de los peces. La gran capacidad filtradora del mejillón y la gran biomasa que pueden alcanzar cuando se cultivan lo convierten en un buen candidato para ser un componente extractivo en un sistema de Acuicultura Multi-Trófica Integrada (AMTI). La AMTI es un concepto que se basa en el reciclado natural, dado que lo desechos producidos por una especie de un nivel trófico elevado son considerados una fuente de alimento para otra especie de un nivel trófico inferior. Un sistema AMTI puede combinar el cultivo de peces con especies extractivas como el mejillón (extracción orgánica) o macroalgas (extracción inorgánica). Actualmente existen diversos estudios que han concluido que el mejillón es capaz de asimilar los desechos de pienso de los peces y mejorar su

crecimiento proporcionando a la vez biorremediación ambiental. Sin embargo, otros estudios han observado que el co-cultivo de peces con mejillón no resulta en un mayor crecimiento del bivalvo. Esta controversia en los resultados pone de manifiesto que los sistemas AMTI son complejos, especialmente cuando se practican en el mar o en las Rías. El mejillón puede ver restringida la ingestión y asimilación de los desechos orgánicos dependiendo de la cantidad de residuos provenientes de las jaulas, la distancia entre las unidades de cultivo de mejillón y las jaulas, la velocidad de la corriente que dispersa las partículas, la biomasa cultivada de ambos niveles tróficos y las características de calidad y cantidad del seston entre otros factores. En este marco conceptual la tesis evaluó si la proximidad a una granja de cultivo de peces incrementa el Scope for Growth (SFG), la cantidad de reservas y el crecimiento en comparación a mejillones cultivados en una batea distante a la granja de peces. El estudio se llevó a cabo en dos escenarios experimentales, la Ría de Ares-Betanzos (España) y la región de Passamaquoddy en la Bahía de Fundy (Canadá). Se integraron parámetros de fisiología energética (tasa de aclaramiento e ingestión, eficiencia y tasa de absorción y costes metabólicos), biometría (longitud de la concha, peso de tejido y de la concha, biomasa), composición proximal (proteínas, carbohidratos, glucógeno y lípidos totales) y biomarcadores moleculares – ácidos grasos e isótopos estables de carbono $\delta^{13}$C) y nitrógeno $\delta^{15}$N). Estos parámetros se midieron junto a las variaciones espacio/temporales de la cantidad y calidad del alimento y de las condiciones físico/químicas del agua.

El estudio en la Ría de Ares-Betanzos se realizó durante cinco campañas experimentales que cubrieron los escenarios hidrográficos representativos de las Rías gallegas: el hundimiento de invierno, el afloramiento de primavera, el periodo de estratificación del verano y el afloramiento del otoño. El muestreo se efectuó en dos bateas de mejillón (*Mytilus galloprovincialis*) del polígono mejillonero de Lorbé. Una de las bateas se encuentra próxima a jaulas de besugo (170 m de distancia) y se sitúa en el interior del polígono. La otra batea se encuentra alejada de las jaulas (550 m de distancia) y en la región exterior del polígono.

El estudio de Passamaquoddy se realizó durante una ocasión en dos sitios de muestreo. En el primer sitio se cultiva mejillón (*Mytilus edulis*) en suspensión inmediatamente adyacente a jaulas de salmón en un sistema AMTI. El segundo sitio consistió en un monocultivo de mejillón situado a 8.5 km de distancia del sistema AMTI.

Las características estacionales del seston en la Ría de Ares-Betanzos reflejaron que durante el afloramiento de primavera y otoño la clorofila-*a* alcanzó los valores más altos (0.48 hasta 1 µg l$^{-1}$) junto con la materia particulada orgánica (MPO; 0.33 hasta 0.5 mg l$^{-1}$). Durante la estratificación térmica de la columna de agua en el verano, la clorofila mostró los niveles más bajos (0.38 a 0.51 µg l$^{-1}$), mientras que durante el hundimiento del invierno, el seston se caracterizó por tener los niveles significativamente más bajos de contenido orgánico (21 a 28 % MPO) en comparación con el resto de las estaciones (41 a 97 % MPO). La mayor cantidad de clorofila registrada en otoño de 2011 (0.67 µg l$^{-1}$) comparado con otoño de 2010 (0.53 µg l$^{-1}$) reflejó la variabilidad inter-anual de la intensidad del viento y la duración de los períodos de estratificación durante el verano. El nanofitoplancton (2-20 µm diámetro) fue la fracción más abundante durante las cinco estaciones, representando el 70% de la clorofila total durante el afloramiento (primavera-verano) y 50 % durante el hundimiento (otoño-invierno). El micro- (>20-50 µm) y picofitoplancton (0.2-2 µm) constituyeron el 13% y 15 % de la clorofila durante el afloramiento y 23% y 24% durante el hundimiento, respectivamente. De igual modo, los mayores y menores contenidos en proteínas, carbohidratos y lípidos del seston fueron registrados en la primavera (44.9%, 17.7%, 16.8 % del peso seco del seston, respectivamente) y el invierno (8.2%, 3.7%, 4.2 % del peso seco del seston, respectivamente). El análisis de la composición de ácidos grasos del seston reveló un alto contenido de marcadores característicos de diatomeas (16:1ω7, 20:5ω3 y ratios 16:1ω7/16:0>1 y 20:5ω3/22:6ω3>1) y bacterias (15:0, 17:0, 18:1ω7 y ratio 18:1ω7/18:1ω9>1) durante el periodo de afloramiento de primavera y verano. Sin embrago, los marcadores de ácidos grasos característicos de dinoflagelados (16:0, 18:1ω9, 18:4ω3, 22:6ω3 y ratio 22:6ω3/20:5ω3>1) predominaron en invierno y otoño. Las sucesiones estacionales de la comunidad fitoplanctónica fueron reflejadas en la composición de

ácidos grasos del manto, la glándula digestiva y las heces de los mejillones de ambas bateas. Además, las variaciones observadas en los parámetros ecofisiológicos, biométricos y bioquímicos medidos en los mejillones de ambas bateas se correspondieron con las fluctuaciones estacionales de la cantidad y calidad del alimento. Se estableció una correlación positiva entre la calidad orgánica del alimento y la clorofila con la tasa de aclaramiento y una correlación negativa con la materia particulada inorgánica (MPI). De este modo, los mejillones aclararon la mayor cantidad de alimento durante la primavera (3 l h$^{-1}$), y se obtuvieron menores tasas de aclaramiento durante los periodos de baja calidad de alimento en invierno (2.4 l h$^{-1}$) y verano (2.9 l h$^{-1}$). El nanofitoplancton fue la fracción que explicó la mayor variabilidad de la tasa de aclaramiento, lo que sugiere que esta fracción es aclarada de forma más eficiente que el micro- y picofitoplancton. La eficiencia de absorción del alimento en el intestino también aumentó con la mayor calidad del seston durante el afloramiento de primavera (97-94 % de eficiencia de absorción), mientras que los valores mínimos fueron durante el hundimiento de invierno (54-34 %). Las elevadas temperaturas registradas durante el periodo de estratificación del verano ocasionaron unas pérdidas metabólicas (respiración y producción de amonio) superiores a las ganancias de energía adquirida a través del alimento. Esto se tradujo en un Scope for Growth negativo durante el verano (-35 J h$^{-1}$). Los máximos valores de SFG se obtuvieron en el afloramiento primaveral (16 J h$^{-1}$), cuando el seston alcanzó un 96% de MPO, siendo la calidad del alimento y la clorofila los factores ambientales que explicaron la mayor variabilidad del SFG. Los resultados del Scope for Growth fueron concordantes con los ciclos de acumulación de reservas metabólicas y las mediciones biométricas. La depleción de las reservas de lípidos y glucógeno de la glándula digestiva y el manto durante el hundimiento de invierno conllevó a una pérdida del rendimiento en carne (1569 mg peso seco) y el SFG (-1 a 4 J h$^{-1}$). Por otro lado, la acumulación de glucógeno en el manto durante los episodios de afloramiento que ocurren de forma intermitente a lo largo del verano, se tradujo en un aumento del peso de la carne del mejillón en el otoño (2358 mg peso seco), lo que fue correspondido con un incremento del SFG (10 a 18 J h$^{-1}$).

Los resultados espaciales obtenidos en la Ría Ares-Betanzos demostraron que los desechos sólidos de los peces (i.e. restos de pienso o productos excretados) no aumentaron significativamente el MPO del seston disponible para los mejillones cultivados en la cercanía de las jaulas de besugo (0.25 a 0.41 mg l$^{-1}$) respecto a los mejillones cultivados a 550 m de la granja de peces (0.30 a 0.58 mg l$^{-1}$). Tampoco se observó un aumento en los niveles de clorofila o de las fracciones de fitoplancton que indirectamente pudieran ser promovidos por los aportes orgánicos e inorgánicos derivados de los desechos metabólicos del cultivo de peces en la batea próxima a las jaulas. El seston mostró una composición bioquímica similar en ambas bateas y no se detectó una incorporación significativa de los marcadores de ácidos grasos característicos del pienso de peces (18:1ω9, 18:2ω6, 18:3ω6, 20:1ω9, 20:4ω6 o ratio ω3/ω6 bajo) en el seston de la batea próxima a las jaulas. Las concentraciones similares de clorofila, calidad y cantidad del seston resultaron en un Scope for Growth, composición bioquímica y crecimiento comparable en los mejillones cultivados en ambas bateas. La falta de un incremento del SFG, cantidad de reservas y crecimiento en los mejillones cultivados en la proximidad a las jaulas de peces puede ser explicados por la interacción de varios factores: las rápidas corrientes de la zona, una distancia demasiado elevada entre las jaulas y la batea y una limitada emisión de residuos orgánicos debido a la baja densidad de besugo cultivada (450 toneladas anuales) en relación a la elevada biomasa de mejillón (promedio anual de 3856 x 10$^3$ mejillones batea$^{-1}$). La mayoría de los estudios que encontraron un aumento en el MPO y los niveles de clorofila en la proximidad de las jaulas se realizaron en zonas con corrientes suaves, en torno a 3-5 cm s$^{-1}$. Sin embargo, es probable que las corrientes presentes en la batea próxima a las jaulas dispersaran los deshechos provenientes del cultivo. Investigaciones realizadas en zonas con corrientes elevadas (3.4 a 24 cm s$^{-1}$) también concluyeron que los desechos metabólicos de los peces como el amonio son transportados demasiado rápido para estimular una mayor producción de microalgas. La localización de la batea a una distancia demasiado elevada respecto a las jaulas (170 m) pudo ser otro de los motivos por el cual no se detectara un aumento significativo de los marcadores de ácidos grasos típicos de las partículas de pienso en el seston. Otros autores han detectado que la mayor concentración de residuos de peces se detecta dentro de los primeros 50

a 60 m de distancia de las jaulas. Además, es probable que la calidad del seston en la Rías gallegas, con un promedio estacional de un 50 % de contenido orgánico, sea óptimo para el crecimiento del mejillón y un aporte extra de alimento en forma de partículas de pienso no cause diferencias perceptibles en el SFG, cantidad de reservas o crecimiento de los mejillones.

Las únicas diferencias espaciales encontradas en la Ría Ares-Betanzos se ciñeron al periodo de invierno y otoño. En dicho periodo, el seston de la batea situada próxima a las jaulas de peces mostró una calidad significativamente menor (38.91 % MPO) que el seston de la batea situada en la parte exterior del polígono (60.52 % MPO). La reducción del MPO se vio reflejada en los menores contenidos de proteínas, carbohidratos y lípidos en el seston de la batea próxima a las jaulas (12.8%, 5.7%,6% del peso seco, respectivamente) comparado con la más alejada (20.5%, 8.7%, 10.5 % del peso seco, respectivamente). Teniendo en consideración que los muestreos de invierno y otoño fueron realizados durante periodos de tormentas, es probable que la resuspensión de las partículas inorgánicas del fondo disminuyera la proporción de partículas orgánicas en suspensión respecto al material inorgánico, y por tanto, la calidad del alimento disponible para los bivalvos. El hecho de que la batea más próxima a las jaulas de peces se encontrara en la región interna del polígono (más próxima a la costa), en una zona menos profunda y con corrientes más elevadas que la batea más alejada, explicaría por qué los fenómenos de resuspensión fueron más intensos en la batea situada en la cercanía de las jaulas. Los eventos de resuspensión resultaron en un aumento significativo de la tasa de aclaramiento que contrarresta la reducción del 20% en la eficiencia de absorción del alimento en los mejillones cultivados en la batea próxima a las jaulas de peces. No obstante, las diferencias espaciales en la calidad de alimento fueron transitorias y en general, se obtuvieron valores semejantes de SFG, composición bioquímica, perfil de ácidos grasos, rendimiento en carne y crecimiento en ambas bateas.

A diferencia de la Ría de Ares-Betanzos, las características del seston en la región de Passamaquoddy de la Bahía de Fundy mostraron diferencias espaciales significativas entre el sistema AMTI y el monocultivo de mejillón. Se observó un contenido significativamente más

alto de clorofila en el sistema AMTI  (3.7 µg l$^{-1}$) que en el monocultivo (2.5 µg l$^{-1}$), aunque esa variación estuvo dentro del rango natural y se necesitaría un muestreo más intensivo antes de confirmar que se trata de un incremento influenciado por los nutrientes de salmón. Los niveles de MPO, proteínas, carbohidratos y lípidos del seston en la zona AMTI (0.5 mg l$^{-1}$, 8.9%, 4.3%, 4.6%, respectivamente) fueron menores que en el monocultivo (0.7 mg l$^{-1}$, 18.8%, 8.4%, 14.4%, respectivamente), ya que se produjeron fenómenos puntuales de resuspensión costeros que disminuyeron la calidad del alimento de forma significativa. Sin embargo, el seston en el sitio AMTI mostró valores significativamente más elevados de los marcadores de pienso 20:1ω9 y 18:2ω6, mientras que el perfil de isótopos estables del seston en ambos lugares también fue diferente. Los mejillones cultivados en el sistema AMTI también asimilaron los marcadores de ácidos grasos del pienso en el manto y la glándula digestiva, que presentaron un alto contenido de 20:1ω9, 18:2ω6, 18:1ω9 y bajo ratio ω3/ω6. El álisis del perfil de ácidos grasos de las heces confirmó que los mejillones utilizan los restos de pienso como una pequeña parte de su dieta. Este resultado remarcó la importancia de establecer sistemas AMTI en ecosistemas con corrientes suaves como las encontradas en este lugar (0-5 cm s$^{-1}$). El cultivo de una menor cantidad de mejillón (1000 mejillones por cuerda) respecto a una gran densidad de salmón (15 kg m$^{-3}$ en jaulas de 8478 m$^{3}$) en comparación a la Ría de Ares-Betanzos (de 1440 x 10$^{3}$ a 4710 x 10$^{3}$ mejillones batea$^{-1}$ y 70 toneladas de besugo totales en la granja durante cada muestreo) también puede haber contribuido a las diferencias. Sin embargo, este aporte extra de alimento en forma de partículas de pienso no fue suficiente para aumentar el SFG y la cantidad de los reservas de los mejillones del cultivo AMTI comparado con los del monocultivo. De hecho, el SFG reflejó la variabilidad espacial en la calidad del alimento. La reducción en la eficiencia de absorción del alimento durante los eventos de resuspensión disminuyó el SFG de los mejillones cultivados adyacentes a la jaula de salmón (28.71 J h$^{-1}$) respecto a los del monocultivo (38.71 J h$^{-1}$). Además, el análisis bioquímico del manto y la glándula digestiva del mejillón reveló una composición similar para ambos sitios de muestreo. Este resultado sugiere que, a largo plazo, los mejillones están expuestos a un alimento de características similares y que los eventos de resuspensión en el sitio AMTI fueron transitorios. Es probable que el aporte extra de alimento a

través de las partículas de pienso de salmón no fuera suficiente para compensar las reducciones puntuales de la calidad del alimento durante la resuspensión de sedimentos, lo que sugirió que los mejillones se alimentan del seston natural principalmente. Es probable que los residuos de pienso del pescado, así como cualquier incremento en los niveles de clorofila inducido por los productos metabólicos de los peces, cobren importancia como un aporte de alimento adicional durante el invierno o el otoño, periodos en los cuales la cantidad y calidad de seston natural es escasa. La integración de los resultados ecofisiológicos, bioquímicos y moleculares sugirió que los ácidos grasos y los isótopos estables son marcadores más sensibles para detectar cambios en la dieta de los mejillones dentro del amplio rango de variación natural del seston. Los marcadores fisiológicos y la composición proximal tuvieron una sensibilidad más limitada para detectar la pequeña asimilación de partículas de pienso de los peces por parte del mejillón cultivado en el sitio AMTI.

En conclusión, los diferentes resultados obtenidos en los ecosistemas de la Ría de Ares-Betanzos y la Bahía de Fundy sugirieron que los mejillones solo asimilan los desechos de pienso cuando son cultivados a una distancia corta respecto a las jaulas de los peces (<170 m), con condiciones de corriente suave y cuando la biomasa de peces de las jaulas es proporcional a la cantidad de bivalvos presentes en el co-cultivo.

# CHAPTER 1

## General introduction

## 1.1. Mussel farming: general status and environmental impact

Aquaculture is usually defined as the culture of aquatic organisms, including fish, molluscs, crustaceans and seaweed. Aquaculture is considered to be the fastest growing food sector in the world with an annual growth rate of 6.3 %, increasing from 34.6 million tonnes in 2001 to 59.9 million tonnes in 2010 (FAO, 2012). The decline in the natural fish stocks and the high demand for seafood of a growing population has lead aquaculture to provide half of the seafood consumed worldwide. The aquaculture production of marine animals reached 18.3 million tonnes in 2010 and mainly comprises the culture of molluscs (75.5 % of the total, 13.9 million tonnes), finfish (18.7 % of the total, 3.4 million tonnes), crustaceans (3.8 %) and other animals (2.1 %) like sea cucumbers and sea urchins (FAO, 2012). Hence, in terms of marine aquaculture, molluscs and fish are considered to be among the most cultured species and their production is expected to keep expanding to support the increasing demand of seafood. Aquaculture produces 11.5 million tonnes of the 20.5 million tonnes of molluscs consumed annually. Bivalve molluscs -mainly mussels, oysters and clams- account for 25 % of the total European aquaculture production (FAO, 2012). Mussels are the second most cultured bivalve molluscs after oysters and The Food and Agriculture Organization estimates that 1,812 thousand tons of mussels worth 1,573 million US$ were farmed in the world in 2010 (FAO, 2012). Mussels are cultured on ropes hung from long-line systems or floating structures like rafts. Spain generates 10% of the total European aquaculture production and mussel *Mytilus galloprovincialis* is the primary product coming from Spanish aquaculture industry. Most of this mussel production comes from the region of Galicia, the second largest mussel producer in the world after China. In Galicia, mussels are cultured in 3,300 floating rafts moored in embayments known as 'Rías' that produce 250,000 tons of mussels per year (Labarta et al., 2004).

Bivalve molluscs are generalist consumers of the particulate organic matter that comprises the seston. The term 'seston' includes phytoplankton, algae detritus, bacteria, protozoa, faecal pellets and mineral particles > 3 mm in diameter. Thus, unlike finfish farming,

the culture of bivalve molluscs is extensive, since it only relies on the natural seston and does not require any additional external food source. Lamellibranchiate or pelecypod bivalves placed in suspended cultivation have access to the whole water column and capture the suspended particles with the ctenidium, a ciliated organ that serves a double function for capturing particles as well as for respiration (Cranford et al., 2011). Water containing oxygen and food particles is draw into the inhalant siphon by the asynchronous motion of the lateral cilia of the gills. Water flows through the ostia (space between the gill filaments) and exits through the exhalent siphon (Cranford et al., 2011). Lastly, bivalves eliminate the undigested organic and inorganic matter in the form of biodeposits. The filter-feeding habits of mussels make them highly sensitive to changes in seston quality and quality. The fluctuations in the feeding environment generally occur at three different scales:

**1) Seagonal changes in phytoplankton production** due to changes in the primary production cycles. The seasonal variations in the quality of the diet for the filter-feeders will be also depend upon the size of the particles suspended in the water column that can be effectively retained by the mussels. The abundance of the different phytoplankton size-classes, pico- (0.2-2 µm diameter), nano- (2-20 µm diameter), and microphytoplankton (>20 µm diameter), rather than the total chlorophyll-*a* biomass, could determine the amount of food available for filter feeders. Even if mussels have the physiological capacity to regulate the retention of particles based on the temporal variations of the food supply, mussels have been demonstrated to have higher retention efficiency for particles within a range of 30 to 35 µm compared to smaller particles of 1 to 15 µm diameter (Strohmeier et al., 2012). The size structure of phytoplankton depends on the concentration of nutrients in the water column, zooplankton grazing and hydrodynamics (Cermeño et al., 2006). Nutrient enrichment of the water during upwelling has been described to enhance the production of large-sized phytoplankton, presumably due to its higher metabolic and growth rate compared to small-sized phytoplankton and also because it is inefficiently grazed by protists (dinoflagellates, ciliates) (Cermeño et al., 2006). In contrast, when nutrients are scarce, small-sized nanoplankton thrives. The Galician Rías have been described to have

two different phytoplankton size structure patterns influenced by the upwelling-downwelling seasons, although intermittency of upwelling can affect phytoplankton species succession. During upwelling large sized microphytoplankton is the most abundant, although nanophytoplankton can also contribute to a large fraction of the total primary production (Figueiras et al., 2002; Cermeño et al., 2006; Froján et al., 2014; Figueiras et al., 2014). Downwelling favourable season will potentially have more picophytoplankton biomass (Figueiras et al., 2002; Cermeño et al., 2006; Froján et al., 2014; Figueiras et al., 2014).

**2) <u>Specific short-term modifications</u>** in the organic fraction of the seston explained by occasional pulses of riverine inputs that swift phytoplankton abundance or by spring/neap tidal cycles (Cranford et al., 2011).

**3) <u>Resuspension of inorganic particles</u>** settled in the seafloor as a result of the local storms (wave or wind-induced resuspension), tides or high current speed. Sediment resuspension often results in a decrease of the food quality. High levels of resuspended inorganic material dilute the organic matter suspended in the water column and reduce the quality of the food available for filter-feeders (Widdows et al., 1979; Hawkins et al., 1999; Wong and Cheung, 2001; Chaparro et al., 2013).

In spite of not needing an exogenous supply of food, the rapid growth of bivalve cultivation has also raised awareness about whether it respects the carrying capacity of the ecosystem, as high crop densities harvest great amounts of phytoplankton. Concerns regarding extensive off-bottom shellfish farming are directed to: i) the reduction or depletion of phytoplankton and ii) the impact that biodeposits -collective term for feces and pseudofeces- and shell debris cause on the benthic communities' structure and composition.

Bivalves and phytoplankton have a reciprocal trophic relationship in which the former exploit the microalgae as a food source and the latter use the organic and inorganic metabolic wastes of the molluscs as nutrients (Newell, 2004). In a system without extensive shellfish culture, phytoplankton cells are usually grazed by zooplankton and benthic feeders and once phytoplankton dies, cells enter the microbial loop and become part of the detritus (Gibbs, 2007).

However, when a suspended bivalve culture is introduced, less phytoplankton would be available for the benthic feeders and zooplankton (Gibbs, 2007). Hence, the abundance of zooplankton will decrease and less would be preyed by pelagic fish. Benthic filter feeders will also decrease, reducing the prey available for demersal fish and creating an ecological niche for benthic deposit feeders (Gibbs, 2007). Shellfish continuous filtering activity has been reported to cause depletion of phytoplankton within rafts up to 57 % which can produce an impact on the pelagic food web and exceed the ecological carrying capacity of the ecosystem (Pérez-Camacho et al., 1995; Navarro et al., 1996; Heasman et al., 1998; Strohmeier et al., 2005; 2008; Petersen et al., 2008). Depletion of phytoplankton assemblages mainly depends on current speed and tidal exchange, natural primary productivity as well as the feeding activity of mussels and the associated epifauna (Cranford et al., 2008; Duarte et al., 2008). Phytoplankton depletion has been also suggested to be size-specific and in high-density mussel culturing areas picophytoplankton (0.2-2 µm) could eventually be the dominant fraction, as it is not effectively retained by mussels (Cranford et al., 2008).

Recent investigations carried out in the Galician Rías estimated that between 10-200 mg of faeces per day are egested by each mussel (Zuñiga et al., 2014). Biodeposits accumulate underneath mussel farms in shallow coastal areas with low hydrodynamism, generating organic enrichment and negative impact in the benthic community beneath the culture units (López-Jamar et al., 1984; Cranford et al., 2009b; Ysebaert et al., 2009). The effects that mussel biodeposits have on the benthos mainly depend on the farm location and the hydrographic conditions, as well as the culturing method and mussel density. Local currents have been considered as one of the most important factor to aid the distribution of the biodeposits (Chamberlain et al., 2001; Hartstein and Rowden, 2004; Zuñiga et al., 2013; 2014). Hartstein and Rowden (2004) proposed to develop mussel farming activities in areas with moderately high current speeds, to prevent alterations on benthic assemblages caused by biodeposits. Concentrating organic matter beneath the rafts alters the food chain, disrupting the energy transfer between the different trophic levels (from bacteria to meiofauna and macrofauna)

(Mirto et al., 2000). Some authors have pointed out that biodeposits have decreased the infaunal diversity in the Galician Rías (López-Jamar et al., 1984), while other organisms of commercial importance such as the velvet swimming crab (*Necora puber*) have proliferated below rafts (Freire and González-Gurriarán, 1995). Ysebaert et al. (2009) developed an interesting study to assess the impact that mussels' biodepositions have on the benthos. They studied two areas with bottom cultures with a micro-tidal (Limfjorden, Denmark) and macro-tidal (Oosterschelde, Netherlands) systems and one area with suspended culture (Ría de Vigo, Galicia) with an upwelling system. The three areas presented high concentrations of phosphorus, phaeopigment and particulate organic carbon and nitrogen. Moreover, a swift in the macrobenthic community was observed, being species typically presented in sandy environments replaced by opportunistic species typically living in enriched sediments.

However, it should be highlighted that mussels' biodeposits induce a lower disturbance than fish farm wastes (Mirto et al., 2000; La Rosa et al., 2002; Crawford et al., 2003; Danovaro et al., 2004), since biodeposits are easily dispersed by the hydrodynamic action and, unlike fish wastes, they mainly increase the levels of nitrogen in the water column (La Rosa et al., 2002; Zuñiga et al., 2013).

## 1.2. Marine fish farming: general status and environmental impact

Fish is one of the most valuable seafood products and the culture of finfish has experience a very fast growth in the last years. For instance, the production of Atlantic salmon increased from 299,000 tonnes in 1990 to 1.9 million tonnes in 2010, at an average annual rate exceeding 9.5 % (FAO, 2010). European mariculture is based mainly on the culture of carnivorous fish like Atlantic salmon, gilt-head bream and seabass in floating net cages. These species account for 75 % of the total European aquaculture production. Sea bream and sea bass are mainly produced in the Mediterranean, while salmon is farmed in Norway and the UK. Considering that the estimated dietary fish requirements in the 21st century will be about 160

million tonnes year$^{-1}$ and that captures from natural fisheries have reached a plateau of 80-90 million tonnes year$^{-1}$, aquaculture has to improve current production to meet this demand. However, in order to maintain its fast growth rate, aquaculture has yet to manage several economical, technological, legal and ecological limitations. Concerns regarding the intensive mariculture of carnivorous finfish are directed towards two main issues: i) the utilization of fish meal and fish oil coming from wild fish stocks as a resource for commercial fish feed manufacturing, and ii) the negative impact that fish waste discharges cause on the marine environment.

Fish meal, fish oil and the so called 'trash fish' coming from the natural fisheries are indispensable for the manufacture of feed, but exert pressure on the wild fish stocks. The high concentration of protein, essential amino acids, lipids, ω3 polyunsaturated fatty acids and minerals present in these products turn them into a perfect raw material for producing fish pellets (Naylor et al., 2000). A second major issue is the loading of great amounts of organic products than can be particulate or dissolved. Particulate products derive mainly from uneaten feed pellets (i.e. large uneaten particles or small feed fines) and fish feces. Dissolved products include excreted ammonia and phosphorous and organic C, N and P through defecation and undigested feed (Wang et al., 2012). Even if direct loss of feed in modern fish farming is considered low, it is estimated that 80-88 % of carbon, 52-95 % of nitrogen and 70-86 % of phosphorous of the total food input gets released into the environment through particulate feed waste, faeces, excreta and respiration (Wu, 1995; Wang et al., 2012). The ecological impact of organic fish products has been widely investigated. The discharge of fish effluents has been associated with organic enrichment of the sediments underneath the net-pens and disruption of seagrass communities (Sarà et al., 2004; Holmer et al., 2007), hypernutrification and phytoplankton blooms near the fish cages (Folke and Kautsky, 1992; Dalsgaard and Krause-Jensen, 2006; Pitta et al., 2009; Sarà et al., 2012). Thus, one of the main challenges that aquaculture has to overcome is to improve the production of fish while reducing the impact that fish effluents have on the environment. The amount of nutrients entering the ecosystem will

depend on the farmed species, stocking density, feeding regimen, digestibility and type of food, as heat-extruded pellets have lower leaching rates than other types of fish feed (Wang et al., 2012). The dispersion of fish waste in the ecosystem would vary depending on the current speed, water depth, resuspension of wastes by bottom currents, the sediment type of the seabed and the rate of consumption and defecation of feed particles by wild fish (Karakassis et al., 2000; Sarà et al., 2004; Holmer et al., 2007). In areas with fast current speeds (10 to 12 cm s$^{-1}$) and 25 m deep, particulate organic wastes have been detected in the sediments at distances up to 1000 m from the fish cages (Sarà et al., 2004). Similarly, sediments near net cages have been found to be enriched in organic carbon, nitrogen and phosphorous at fish farms with rapid current speeds (>5.5 cm s$^{-1}$) and fast water exchange (Holmer et al., 2007). Even if the sedimentation of fish waste declined with distance, fish effluents were still detected at a distance of 40 m from the cages and waste products were not only dispersed along the prevailing current direction (Holmer et al., 2007). In semi-enclosed bays with slower current speed (3.5 cm s$^{-1}$), fish wastes get less dispersed and most of the organic enrichment was found between 0 to 10 m from fish cages, were sediments showed two times higher C and N content than control sites (Karakassis et al., 2000). In contrast, only a slight organic enrichment of the sediments was found at 5 m distance from fish farms located in regions with strong current speeds (6.3 cm s$^{-1}$) (Karakassis et al., 2000).

## 1.3. Integrated Multi-trophic Aquaculture (IMTA)

Integrated Multi-Trophic Aquaculture (IMTA) has been proposed as a possible solution to mitigate the particulate organic solids and dissolved wastes coming from fish cage farming (Troell et al., 2003; 2009; Neori et al., 2004). IMTA is originally based on the traditional polyculture systems originated in Asia, where domestic carp farming was performed next to rice fields. The main difference between polyculture and IMTA is that the former consists of the culture of different species from the same trophic level (i.e. different species of carp in the same pond), while the later combines the culture of different trophic levels. Thus, IMTA could

combine feed aquaculture species (i.e., fish and crustaceans such as shrimp) with organic extractive species (e.g. molluscs, sea urchins, sea cucumbers) and inorganic extractive species (e.g. seaweed) (Neori et al., 2004; Buschmann et al., 2009; Troell et al., 2009). IMTA is a natural recycling concept in which nutrient discharges from one species are considered nutritional inputs for another. The connotations of the term integrated imply that the different trophic levels are grown adjacent to each other to allow the transference of surplus energy from higher to lower trophic levels, diversifying the production and, most importantly, achieving a more sustainable culture (Barrington et al., 2009).

Intensive fish farming in cages located in the sea produces both a direct and indirect enrichment of the total particulate matter (TPM) at the pelagic and benthic environment. The direct contribution of fish is made by carbon, nitrogen and phosphorus in the form of unconsumed pellets and fish faeces. The indirect contribution is through nitrogenous waste (ammonia and urea) and phosphorus that are dissolved in the water column. Large particles of uneaten feed and faeces can sink to the bottom and be consumed by bacteria or other benthic organisms (e.g. urchins, sea cucumbers), while small particles in suspension could be potentially taken up by filter-feeders such as mussels (Troell and Norberg, 1998; Cranford et al., 2013). The dissolved nutrients could also be assimilated by phytoplankton surrounding the fish cages (Chopin et al., 2008; Troell et al., 2003; 2009) and might induce chlorophyll blooms that can benefit the co-cultured bivalves (Jones and Iwama, 1991; Stirling and Okumus, 1995; Dalsgaard and Krause-Jensen, 2006; Pitta et al., 2009; Sarà et al., 2011; 2012).

In recent years, the possibility of growing seaweed with fish (Neori et al., 2004; Chopin et al., 2008), bivalves with fish (Stirling and Okumus, 1995; Mazzola and Sarà, 2001) and fish with crustaceans (Troell et al., 1999) has been investigated. Mussels are one of the main organic extractive components used in open-water IMTA systems. Mussels are cultured in IMTA systems using mesh socks or ropes that are hung from rafts or long-lines (Cranford et al., 2013). Mussels have several traits that make them ideal candidates for fish wastes biomitigation: they are edible species with a wide geographic distribution; they can clear water with high efficiency

and even deplete the phytoplankton production as explained in section 1.1.; and can be cultured in dense populations and at relatively high biomass (Cranford et al., 2013). However, the scientific literature confronts a controversy between studies that concluded that fish-bivalve culture can be developed successfully (Wallace, 1980; Jones and Iwama, 1991; Lander et al., 2004; Chopin et al., 2008; MacDonald et al., 2011; Aguado-Giménez et al., 2013) to other investigations that didn't observe any (Taylor et al., 1992; Parsons et al., 2002; Cheshuk et al., 2003; Navarrete-Mier et al., 2010) or almost any (Stirling and Okumus, 1995) enhancement in bivalves' growth when cultured adjacent to teleost fish. These results highlight that the successful implementation of mussel-fish IMTA systems is complex, as the capture of fish particles by the mussel ctenidium may be constrained by the amount of effluent coming from the fish cages, distance between the fish and the mussels, the current speed of the site, bivalve and fish cultured biomass, the response time of the local primary production to the excess nutrients and temporal and spatial variations in ambient seston concentrations and seston organic content among other factors (Cheshuk et al., 2003; Troell et al., 2011; Cranford et al., 2013).

The potential capacity of mussels to biomitigate organic fish wastes has been analyzed using several ecophysiological, biochemical and molecular indicators. Previous studies have investigated mussel feeding and digestive physiological rates. *Mytilus edulis* and *M. trossulus* absorption efficiency (Reid et al., 2010) and *Mytilus edulis* clearance rate, filtration rate and exhalent siphon area (MacDonald et al., 2011; Handå et al., 2012a) were enhanced when mussels were exposed to salmon feed and feces waste particles. Other authors have measured mussel growth using biometric parameters (shell length, tissue and shell weight) and the condition index (CI) that estimates the mussel meat yield at IMTA sites relative to control individuals (Taylor, 1992; Cheshuk et al., 2003; Peharda et al., 2007; Lander et al., 2012). Other authors have analyzed the proximate biochemical composition of mussels growing near fish cages or exposed to fish effluents, to assess if the surplus organic particles enhanced the protein, carbohydrates or lipid levels of the mussels' tissues compared to a control diet (Taylor et al.,

1992; Both et al., 2011; 2012, 2013). Certain fatty acids (FA) characteristic of fish feed have been proposed as biomarkers for demonstrating fish feed waste uptake by mussels. Vegetable terrestrial oils are being utilized for fish feed manufacturing as an alternative to reduce the expenditure of fish oil. As a consequence, fish pellets have a high content of 18:1ω9, 18:2ω6, 18:3ω6, 20:1ω9, 20:4ω6 and a low ω3/ω6 ratio (Gao et al., 2006; Redmond et al., 2010; Both et al., 2011; Handå et al., 2012a; b). These FA are considered potential biomarkers because they are not found in marine food webs and have been demonstrated to be present in significantly higher proportions in mussels fed with fish effluents in comparison with control diets (Gao et al., 2006; Redmond et al., 2010; Both et al., 2011; Handå et al., 2012a; b). Lastly, carbon ($\delta^{13}$C) and nitrogen ($\delta^{15}$N) stable isotopes (SI) have been previously used to trace the incorporation of uneaten trash fish or particulate fish waste into the mussels' tissues (Gao et al., 2006; Xu and Yang, 2007; Redmond et al., 2010). Heavier isotopes $^{13}$C and $^{15}$N are incorporated in the consumers' tissues, since lighter isotopes ($^{12}$C and $^{14}$N) are lost: i) through excretion ($^{14}$N) and respiration ($^{12}$C), ii) through the preferential uptake of $^{13}$C and $^{15}$N enriched compounds during digestion and assimilation or, iii) through fractionation during synthesis of different tissues (Michener and Kaufman, 2007). Thus, the carbon and nitrogen isotopic compositions of consumers reflect their diet with enrichment in heavier isotopes. This enrichment in heavier isotopes is known as trophic shift (Michener and Kaufman, 2007). The $\delta^{15}$N stable isotope ratio usually enriches by 3-4‰ per trophic level, hence $\delta^{15}$N can be used to define the trophic position of each organism in a food web (Michener and Kaufman, 2007). The stable $\delta^{13}$C isotope ratio usually increases by 1‰ per trophic level; therefore it can be used to track primary carbon sources for higher trophic levels (Michener and Kaufman, 2007).

The aforementioned physiological, biochemical and molecular indicators were used as a tool throughout this thesis to study if mussels cultured near fish farms were effectively assimilating fish feed wastes in two different scenarios, the Ría Ares-Betanzos (Galicia, Spain) and the Passamaquoddy region of the Bay of Fundy (Canada).

The Galician coastline (10° to 44° N) consists of 18 Rías that form a unique littoral system in the Atlantic coast and cover 25% of the Iberian Peninsula. The high primary production of the Galician Rías is attributed to the continuous succession of the upwelling-downwelling oceanographic seasons and to its coastal morphology. Upwelling occurs in spring-summer (March to October) when the Azores High pressure cell moves northward and north-easterly winds transport the Ekman layer along the continental shelf (Figueiras et al., 2002; Álvarez-Salgado et al., 2011). On the other hand, downwelling occurs in autumn-winter, when the Greenland Low intensifies and wind blows from the south, reversing the circulation pattern of the Ría (Figueiras et al., 2000). The standard residual circulation pattern of the Rías consists of an outflowing surface current enriched in continental runoff and an ingoing bottom current enriched in shelf waters (Peteiro et al., 2011). When northerly winds are dominant this circulation pattern enhances. However, when southerly winds intensify the pattern can lessen or even reverse. The Ría of Ares-Betanzos is located on the north coast of Galicia. Mussel production in Ría de Ares-Betanzos is 10,000 tonnes year $^{-1}$ (Labarta et al., 2004) and most rafts are placed within the Lorbé raft Polygon (147 rafts). All the experiments conducted in Ares-Betanzos were based in Lorbé (Latitude 43° 23' 24.74" N, Longitude 8° 17' 48.30" W) in two rafts used for commercial mussel cultivation known as P-14 and P-46. Raft P-14 was placed in the inside area of Lorbé, in the proximity (170 m North) of a red sea bream (*Pagellus bogaraveo*) fish farm. Raft P-46 was moored in the outside of the Bay, 550 m North from the net pens. The experiments were conducted during five different seasons at both mussel rafts.

The second study site was based in the Passamaquoddy region of the Bay of Fundy, on the east coast of Canada. The Bay of Fundy is a large body of water located between New Brunswick and Nova Scotia, at approximately 44º and 46º N latitude. The Passamaquoddy Bay is an inlet part of the Bay of Fundy and has a very large tidal range (≥ 6 - 8 m) and high tidal currents velocities, due to the combination of the semidiurnal tide of the North Atlantic Ocean with the topography of the Bay of Fundy (Gregory et al., 1993; Wildish et al., 1993). Passamaquoddy is the main region for Atlantic salmon (*Salmo salar*) mariculture within the Bay

of Fundy, with 25,000 tons year-1 (~25 % of the total Canadian production) worth 159 million US$ (Statistics Canada, 2010). Salmon mariculture was first introduced in 1979 in Deer Island and nowadays is cultured in more than 93 farms that provide more than 2,000 direct and indirect jobs (Walters, 2007). The production of blue mussel *Mytilus edulis* is not fully established in the Bay of Fundy, owing to the seasonal occurrence of toxic phytoplankton blooms that have negative effects for human health (Lander et al., 2004). All the experiments conducted in the Bay of Fundy were performed in two sites: a mussel raft moored adjacent to a salmon pen in Deer Island and a monoculture mussel farm placed 8.5 km away from the salmon farm. All the experiments were conducted during one sampling occasion at both sites.

## 1.4. Objectives

The general aim of this thesis was to study the bioenergetics, biochemical composition and growth of mussels reared in the proximity of fish cages in comparison with the performance of bivalves cultured in locations further away from the fish pens.

To examine the performance of mussels cultured near fish cages, this thesis aimed to accomplish the following main objectives:

**1.** To understand the characteristics of the seston, chlorophyll-*a*, and phytoplankton size-classes available on a seasonal basis for mussels cultured in suspension in sites near and distant from fish cages. To study if fish solid and dissolved wastes increased the organic content of the seston and the chlorophyll levels near the fish cages.

**2.** Assess the effect of seasonal and spatial fluctuations in seston quality and quantity on the bioenergetic responses of mussels cultured at the sites close and distant from the fish cages. The effects of the variable feeding environment were investigated on the different physiological parameters that integrate the Scope for Growth: feeding rates (clearance rate and organic

ingestion rate), digestive rates (absorption efficiency and absorption rate) and metabolic rates (oxygen consumption and ammonia excretion rate).

**3.** Determine the temporal and spatial shifts of the gross biochemical composition (proteins, carbohydrates and lipid content) and fatty acid signatures of the seston and mussels cultured close and further away from the fish farm.

**4.** To investigate the assimilation of fish feed fatty acid biomarkers by mussels suspended near the fish farm and to compare the isotopic profile of bivalves cultured at both sites.

**5.** Study the growth and Condition Index of mussels held in proximity to the fish cages and compare these biometric parameters with those obtained for mussels growing further away from the fish pens.

# Chapter 2

Absorption efficiency of mussels *Mytilus edulis* and *Mytilus galloprovincialis* cultured under Integrated Multi-Trophic Aquaculture conditions in the Bay of Fundy (Canada) and Ría Ares-Betanzos (Spain)

## ABSTRACT

Integrated Multi-Trophic Aquaculture (IMTA) is a recycling concept in which waste nutrient discharges from high trophic levels become an additional energetic input for extractive organisms such as bivalves. The aim of this study was to measure the seston levels and absorption efficiency of mussels reared in the proximity of fish net-pens. The absorption efficiency of mussels *Mytilus galloprovincialis* and *Mytilus edulis* cultured at sites adjacent to red sea bream (*Pagellus bogaraveo*) and salmon (*Salmo salar*) cages was assessed on site, using natural seston diets and compared with mussels reared distant from the cages in the Ría de Ares-Betanzos (Galicia, N.W. Spain) and the Bay of Fundy (S.W. New Brunswick, Canada), respectively. Total particulate matter and the organic and the inorganic fractions of the seston were measured simultaneously. Seston parameters were generally similar at the mussel sites close to the fish cages and at the reference sites. However, significantly higher particulate inorganic matter coupled with lower food quality (seston organic content) observed at the sites close to the fish cages suggested occasional sediment resuspension events in the Ría de Ares-Betanzos and the Bay of Fundy. Owing to the reduced food quality, 20 % lower absorption efficiency was measured for mussels in the proximity to the cages during the resuspension events. No significant differences in absorption efficiency were detected between the fish cages and the reference sites outside the resuspension events. Consequently, differences in absorption efficiency were attributed to natural variations in seston organic content, and absorption increased with increasing food quality. The results showed no evidence of increased organic content of the seston resulting from proximity to the fish-farm. It was concluded that proximity of cultured mussels to the fish cages did not result in an enhancement of the absorption efficiency.

*Key words: Integrated Multi-Trophic Aquaculture; Seston quality; Absorption Efficiency; Mytilus galloprovincialis; Mytilus edulis.*

# 1. Introduction

The Galician coastline has a unique system of flooded river valleys called 'Rías' that benefit from cold nutrient-rich waters during the upwelling season and provide a sheltered area very suitable for the suspended culture of mussels. The raft culture of the mussel *Mytilus galloprovincialis* in Galicia produces 250,000 tons year $^{-1}$ and is the main mariculture industry of Spain, and one of the most important in Europe (Labarta et al., 2004). During recent years fish-farming in floating cages has also been introduced in the Galician Rías, where space to allocate new culture facilities is already limited. One possible consequence of fish culture is that the discharge of unconsumed feed pellets and fish faeces could lead to the eutrophication in the culture region. In areas with extensive fish aquaculture like the Bay of Fundy (SW New Brunswick, Canada), these conflicts have been addressed with the simultaneous culture of Atlantic salmon (*Salmo salar*), blue mussels (*Mytilus edulis*) and kelp (*Laminaria saccharina* and *Alaria esculenta*). These Integrated Multi-Trophic Aquaculture (IMTA) sites are currently working at a commercial pilot scale (Troell et al., 2009; Reid et al., 2010; MacDonald et al., 2011; Liutkus et al., 2012). The filter-feeding capacity of bivalves has been proposed as possible means of significantly reducing the organic effluents released from open-water fish farms. In these IMTA systems, the energy loss from the fed trophic level (caged fish) becomes an energy input for mussels, which have been reported to grow up to 50 % higher when cultured in proximity to fish cages than at control sites in the Bay of Fundy (Troell et al., 2009). Mussel culture in coastal areas like the Galician Rías and the Bay of Fundy are subjected to large natural temporal and spatial fluctuations in seston quantity and quality as a consequence of the seasonal cycle of primary production, the upwelling-downwelling cycle in the Galician Rías, horizontal phytoplankton patchiness, and storm or tide-induced resuspension of bottom sediments (Figueiras et al., 2002). Fish effluents may represent an additional source of particulate organic matter for shellfish, particularly when ambient seston levels are low (Troell et al., 2003; Cheshuk et al., 2003; Neori et al., 2004; 2007; Barrington et al., 2009).

The research on the effects of particulate organic fish effluents on the growth of bivalves has increased rapidly in recent years. Many studies have revealed that mussels and oysters grew faster and were able to uptake the organic waste when co-cultured with fish (Wallace, 1980; Jones and Iwama, 1991; Buschmann et al., 2000; 2009; Lefebvre et al., 2000; Mazzola and Sarà, 2001; Lander et al., 2004; Peharda et al., 2007; Chopin et al., 2008; Sarà et al., 2009; Reid et al., 2010; MacDonald et al., 2011; Sarà et al., 2012; Handå et al., 2012a). On the other hand, some authors did not detect any significant growth enhancement in similar experiments integrating fish with bivalves (Taylor et al., 1992; Stirling and Okumus, 1995; Gryska et al., 1996; Parsons et al., 2002; Cheshuk et al. 2003; Navarrete-Mier et al., 2010). Two recent studies have focused on the absorption efficiency (AE) of mussels cultured under IMTA conditions (Reid et al., 2010; MacDonald et al., 2011) but knowledge of the AE of fish effluents remains limited. Effluent particle size, organic content and biochemical composition have been reported to be the most important factors determining food absorption and growth of co-cultured bivalves (Reid et al., 2009; Both et al., 2013). *Mytilus edulis and M. trossulus* absorption efficiency (Reid et al., 2010) and *M. edulis* absorption efficiency, clearance rate, filtration rate and exhalent siphon area (MacDonald et al., 2011) were reported to be enhanced when exposed to salmon feed and faeces particles under laboratory conditions. Further study of particulate fish effluent availability and the digestive capabilities of waste extractive species such as mussels may help to explain the inconsistency in bivalve growth responses at IMTA sites noted above.

In this study we investigated the absorption efficiency of two different species of mussels cultured in the proximity of fish farms. Two field experiments were carried out to study the seston characteristics and the absorption efficiency of mussels *M. galloprovincialis* and *M. edulis* cultured in the proximity of red sea bream and Atlantic salmon farms in Galicia and New Brunswick, respectively. The objective of this study was to compare the absorption efficiency of *M. galloprovincialis* held in suspended cultivation in rafts located both distant and close to red sea bream (*Pagellus bogaraveo*) floating cages in the Ría of Ares-Betanzos (Galicia, NW Spain) during five seasonal surveys. Seston quality and quantity were also compared between two mussel rafts. This is the first study in which these comparisons have been made under extensive,

commercial-scale, mussel culture conditions. A significant and biologically meaningful increase in the AE of the cultured mussels would represent an additional source of income for Mytiliculture in Galicia, where integrated mussel-fish culture has not been investigated. In addition, comparisons between seston parameters and mussel absorption efficiency were performed on one sampling occasion at a commercial *M. edulis*-salmon (*Salmo salar*) IMTA system in the Bay of Fundy, Canada. IMTA in this area has been more extensively studied but additional work is needed to substantiate the ability of the IMTA concept to both increase long-term aquaculture sustainability and profitability.

# 2. Material and methods

## 2.1. Study site

The study was carried out in two separate regions. The first experimental site was in the Ría de Ares-Betanzos (Galicia, NW Spain) at the Lorbé mussel raft polygon (43°23'24.74''N; 8°17'48.30''W) (Fig. 1 A). The Ría Ares-Betanzos is a V-shaped inlet divided in two parts: an inner shallower part consisting of the estuaries of river Eume and Mandeo, and an outer deeper part that is connected to the shelf (Álvarez-Salgado et al., 2011). Annual river discharge is 30 $m^3 s^{-1}$ and the importance of continental runoff and nutrient discharges by the rivers is greater than in the Rías Baixas (Álvarez-Salgado et al., 2011). The Ría is a partially stratified estuary with strong vertical mixing and it has a mesotidal and semidiurnal tidal cycle that ranges from 0.02 to 4.14 m during neap and spring tides, respectively (Sánchez-Mata et al., 1999; Álvarez-Salgado et al., 2011). North-easterly winds induce upwelling events from March-April to September-October (spring-summer), while south-westerly winds induce downwelling the rest of the year (Figueiras et al. 2002; Álvarez-Salgado et al., 2011). Seasonality of rainfall in Galicia ranges from moderate to dry during upwelling (average rainfall of 80 and 30 mm in May and July) to strong during downwelling (average rainfall of 110 and 102 mm in October and February) (AEMET, 2012). Frequent storms from the Atlantic Ocean hit the Galician coast during autumn and winter and are characterized by high waves that induce the mixing of the

water column. The Lorbé raft polygon is situated in the southern shore of the Ría Ares-Betanzos (between 10 and 30 m) and is the main area of mussel *Mytilus galloprovincialis* culture, with 107 rafts and a total production of 10,000 tons year[-1] (Labarta et al., 2004). The seabed in Lorbé is dominated with medium to fine sand with organic carbon contents < 2.8 %, indicative of high energetic conditions (Sánchez-Mata et al., 1999). The organic content of the sediments is influenced by anthropogenic inputs from sewage and industrial waste, local eutrophication by extensive mussel raft culture and continental runoff from rivers Eume and Mandeo (Sánchez-Mata et al., 1999). The inorganic fraction of the sediments is rich in silt and clay (Sánchez-Mata et al., 1999). Average current velocity in Lorbé raft polygon is 2-3 cm s^{-1} and the variance of the current direction has been shown to be dominated by the tide (Piedracoba et al., 2014).

Sampling was conducted at two commercial rafts within Lorbé polygon. Raft P-14 was situated in the inner region of the polygon and 170 m north from red sea bream (*Pagellus bogaraveo*) net cages. Raft P-46 was used as a reference site and was located in the outer region of the polygon, 550 m away from the net-pens. The rafts had a surface area of 550 m[2] (25 x 22 m) containing a maximum of 500 hanging ropes with a length of 12 m. Rafts were anchored by a single chain placed on one side to enable the raft to face their frontal side into the main current entering the polygon. Raft P-14 was anchored at 14 m depth while raft P-46 was at 16 m.

The red sea bream farm was located in the inner Lorbé region and consisted of 48 circular floating net cages arranged in parallel rows. The cages were 28 m in diameter with a net depth of 6 m and a total volume of 3,692.64 m[3], with a stocking density of about 10 kg m^{-3}. The approximate annual production was 245 tonnes of sea bream (JACUMAR, 2011). Sea bream are kept in the cages for three years until reaching a commercial weight of 0.8 kg.

The second experiment was performed in the Bay of Fundy (SW New Brunswick, Canada) at two different sites. First, the sampling was conducted at Clam Cove Atlantic salmon (*Salmo salar*) farm (44°57'52.2''N; 67°0'46.65''W) near Deer Island (Passamaquoddy Bay) (Fig. 1 B). Mussels (*Mytilus edulis*) are co-cultured in suspension next to salmon sea-cages at a pilot commercial scale. The culture site is situated within 150 m from the coast. The salmon farm consisted of 20 circular cages that were approximately 30 m in diameter and 12 m of net depth

(total volume 8,478 m$^3$), with a stocking density of 15 kg m$^{-3}$. Salmon are kept in cages for 16 months until they reach 4 kg.

The second site (reference) was a mussel farm situated 8.5 km distant from Deer Island (Lease MF 377: 45°02'58.2''N; 67°01'55.43''W) where *M. edulis* are reared following the standard (non-IMTA) long-line commercial protocols. Bivalves used in this experiment were randomly obtained from a long-line on MF 377.


## 2.2. Experimental design

Environmental and physiological measurements at the two Lorbé mussel rafts in Galicia were obtained over two consecutive days, during five seasonal campaigns in the summer (July 2010), autumn (October 2010 and 2011), winter (February 2011) and spring (May 2011) in Galicia. The two sampling periods in October were executed to test for inter-annual variance. The experiment in the Bay of Fundy was performed over two days in a single campaign in the summer (June 2011). The absorption efficiency experiments were conducted onboard a moored boat to maintain ambient conditions of temperature, salinity, and food availability. Seawater from 3 m depth was supplied by a peristaltic pump to a header tank and filtered through a 50 µm nylon mesh before being distributed at a constant flow rate to the experimental chambers (Filgueira et al., 2006). On each visit, 15 mussels of 50-60 mm shell length were collected from 3 m depth and placed in 250 ml individual chambers supplied with flowing seawater. Mussel shells were cleaned of epibiotic organisms before being placed in the chambers. Seawater samples were collected from the outflow of an empty chamber during the absorption efficiency experiments to characterize the natural seston.

**A)**
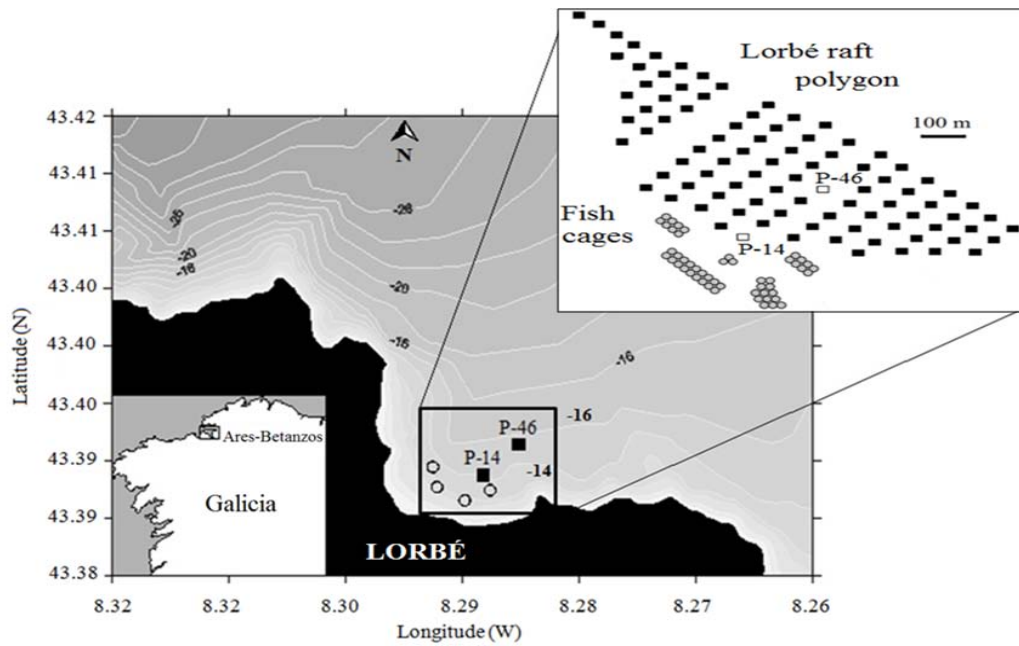


**B)**



Fig.1. Maps of the two experimental sites in (A) the Lorbé mussel raft polygon situated in the Ría of Ares-Betanzos (Galicia, NW of Spain) and (B) the Bay of Fundy (SW New Brunswick, Canada). Open circles indicate the position of the fish cages and squares indicate the position of the mussel rafts in Lorbé. The mussel long-line site (MF 377) and Clam Cove site in Deer Island are indicated with a star and a circle, respectively.

## 2.3. Environmental parameters

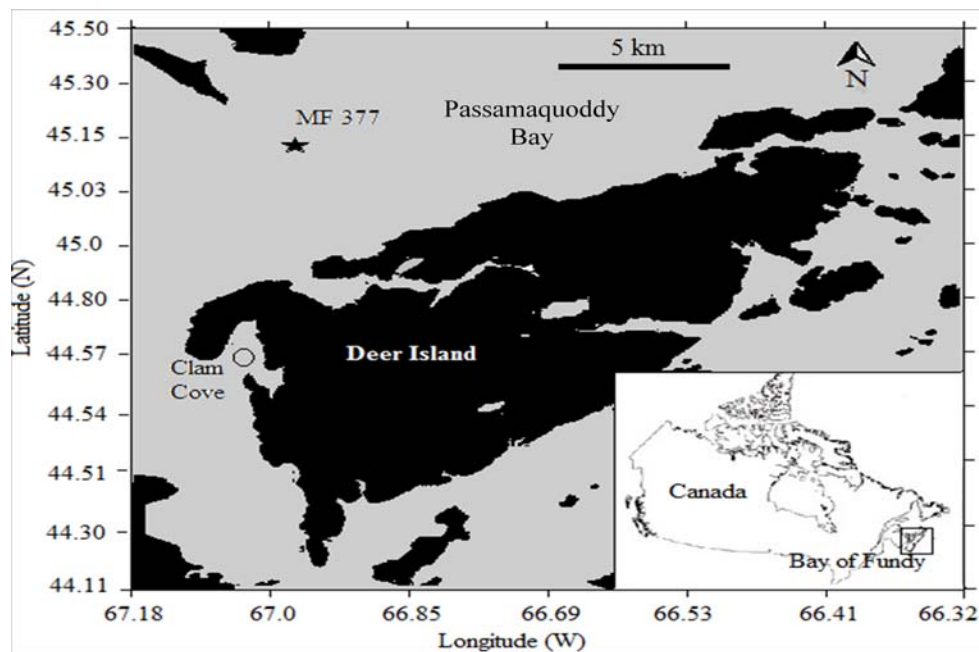The quality and quantity of seston was determined on each sampling campaign as follows. Total particulate matter (TPM; mg l$^{-1}$) and the constituent organic (POM; mg l$^{-1}$) and inorganic (PIM; mg l$^{-1}$) concentrations were gravimetrically determined. Seston samples were filtered onto pre-ashed (450 °C for 4 h) and pre-weighed Whatman GF/F filters and rinsed with isotonic ammonium formate (0.5 M) to remove salts and prevent lysing of living algal cells. TPM was determined as the weight increment after drying the filters to constant weigh at 110 °C. Filters were then ashed at 450 °C in a muffle furnace to determine the content of PIM. Particulate organic matter corresponded to the difference between the total dry mater weight and the ash weight. Filters were weighed with an accuracy of 0.001 mg using an electronic microbalance (Sartorius M3P, M3P-000V001). Seston quality was expressed as $Q_1$ = POM / TPM to account for the relative organic content by weight.

## 2.4. Absorption efficiency (AE)

Mussels were initially left undisturbed for 1 h after being sampled from the ropes. Faeces produced by each mussel after this acclimation period were collected after 4 h. Representative samples of the diet were collected during the absorption efficiency experiments (see above). Faeces samples were filtered through pre-combusted and pre-weighed Whatman GF/F filters. Filters were washed with ammonium formate and dried at 80 ºC until constant weight, weighed and combusted at 450 ºC for 3 h. Filters were weighed to determine the faeces organic and inorganic content as described for the seston samples. Absorption efficiency (AE; %) represents the effectiveness with which material cleared from suspension is absorbed during passage through the digestive system. AE was calculated following the method of Conover (1966):

$$AE = [(F-E) / (1-E) F] \times 100 \tag{Eq. 1}$$

in which F and E are the percentage organic contents (by weight) of seston and faeces, respectively.

## 2.5. Data analyses

A two-way analysis of variance (ANOVA) was performed for the environmental and physiological parameters obtained at the Lorbé mussel rafts polygon to test for significant effects of the factors site and season. A one-way ANOVA was employed to test for differences in mean values of the AE and seston among mussels cultured in the two sites sampled in the Bay of Fundy. The assumptions of normality and homogeneity of variance were tested with Shapiro-Wilk and Levene tests, respectively. A non-parametric ANOVA by ranks was performed when data did not conform to the assumptions of normality and homogeneity of variance. Tukey's HSD *post-hoc* test was selected for pairwise comparisons on main significant effects. Pearson's correlation coefficients were computed to identify the significant relationships existing between the environmental parameters and the absorption efficiency of mussels cultured in Lorbé. Backwards multiple regressions were performed including all the variables that had a significant relationship with the absorption efficiency to identify the independent variable explaining the highest variability.

All data analyses were performed using Statistica 7.0 (StatSoft, Inc.). Statistically significant differences were considered at P-values < 0.001.

## 3. Results

### 3.1. Environmental conditions

The mean seston values and the standard deviation (SD) recorded throughout the complete experimental period at the two Lorbé mussel rafts are shown in Figure 2. Average total particulate matter (TPM) levels varied seasonally. TPM present at the raft distant from the fish cages (n = 31) varied over a range of 0.38-0.52 mg l$^{-1}$ during the spring-summer period and from 0.41-1.08 mg l$^{-1}$ during the autumn-winter (see Table 1). Values of TPM (n = 29) registered at the raft close to the cages were 0.44-0.53 and 0.63-2.36 mg l$^{-1}$, respectively. Average TPM values were $0.60 \pm 0.22$ mg l$^{-1}$ and $1.04 \pm 0.70$ mg l$^{-1}$ for P-46 and P-14, respectively. Levels of POM and PIM computed at the raft distant from the cages ranged from

0.25-0.41 and 0.004-0.87 mg l$^{-1}$ during the complete experimental period, while values at the raft in proximity (n = 29) to the cages ranged from 0.30-0.58 mg l$^{-1}$ and 0.004-1.98 mg l$^{-1}$, respectively (Table 1).

| Site | Season | AE | TPM | POM | PIM | $Q_1$ |
|---|---|---|---|---|---|---|
| | | % | mg l$^{-1}$ | mg l$^{-1}$ | mg l$^{-1}$ | |
| **Lorbé** | | | | | | |
| P-46 | Summer | 77.19 ± 4.07 | 0.52 ± 0.04 | 0.35 ± 0.01 | 0.17 ± 0.04 | 0.67 ± 0.05 |
| P-14 | 2010 | 77.78 ± 1.77 | 0.53 ± 0.07 | 0.37 ± 0.04 | 0.15 ± 0.03 | 0.70 ± 0.03 |
| P-46 | Autumn | 77.78 ± 1.67 | 0.51 ± 0.04 | 0.33 ± 0.03 | 0.18 ± 0.01 | 0.64 ± 0.01 |
| P-14 | 2010 | 66.55 ± 2.30 | 0.63 ± 0.07 | 0.33 ± 0.02 | 0.30 ± 0.05 | 0.53 ± 0.03 |
| P-46 | Winter | 54.78 ± 1.25 | 1.08 ± 0.16 | 0.30 ± 0.03 | 0.77 ± 0.13 | 0.28 ± 0.02 |
| P-14 | 2011 | 34.92 ± 1.82 | 2.36 ± 0.11 | 0.50 ± 0.00 | 1.86 ± 0.10 | 0.21 ± 0.00 |
| P-46 | Spring | 97.51 ± 0.26 | 0.38 ± 0.03 | 0.37 ± 0.03 | 0.01 ± 0.00 | 0.97 ± 0.01 |
| P-14 | 2011 | 94.60 ± 0.82 | 0.44 ± 0.01 | 0.42 ± 0.01 | 0.02 ± 0.00 | 0.95 ± 0.02 |
| P-46 | Autumn | 91.75 ± 0.42 | 0.41 ± 0.04 | 0.36 ± 0.01 | 0.05 ± 0.02 | 0.88 ± 0.05 |
| P-14 | 2011 | 64.43 ± 2.73 | 1.23 ± 0.21 | 0.50 ± 0.04 | 0.72 ± 0.17 | 0.41 ± 0.02 |
| **Bay of Fundy** | | | | | | |
| MF 377 | Summer | 69.44 ± 3.60 | 1.68 ± 0.10 | 0.75 ± 0.03 | 0.93 ± 0.09 | 0.45 ± 0.02 |
| Clam Cove | 2011 | 55.64 ± 2.92 | 1.99 ± 0.11 | 0.54 ± 0.01 | 1.45 ± 0.10 | 0.27 ± 0.01 |

Table 1. Mean values ± SD of absorption efficiency (AE; %) and environmental parameters registered seasonally at the two study sites, Lorbé raft polygon (Galicia, N.W. Spain) and The Bay of Fundy (S.W. New Brunswick, Canada). The sites distant from the fish cages are denoted as raft P-46 and long-line MF 377. The culture unit in the vicinity of the cages is referred as P-14 and the site with the integrated culture as Clam Cove. Seston total particulate matter (TPM; mg l$^{-1}$), organic (POM; mg l$^{-1}$) and inorganic (PIM; mg l$^{-1}$) fractions values are also shown. Seston quality index was expressed as $Q_1$ = POM / TPM to account for the relative organic content by weight.

Fig. 2. Box- plots representing the mean seasonal values of A) total particulate matter (TPM; mg l $^{-1}$); B) particulate organic matter (POM; mg l $^{-1}$); C) particulate inorganic matter (PIM; mg l $^{-1}$); D) $Q_1$ (POM / TPM) registered at the rafts distant (P-46) and close (P-14) to the fish cages. Significant differences are denoted by $P < 0.001$***.
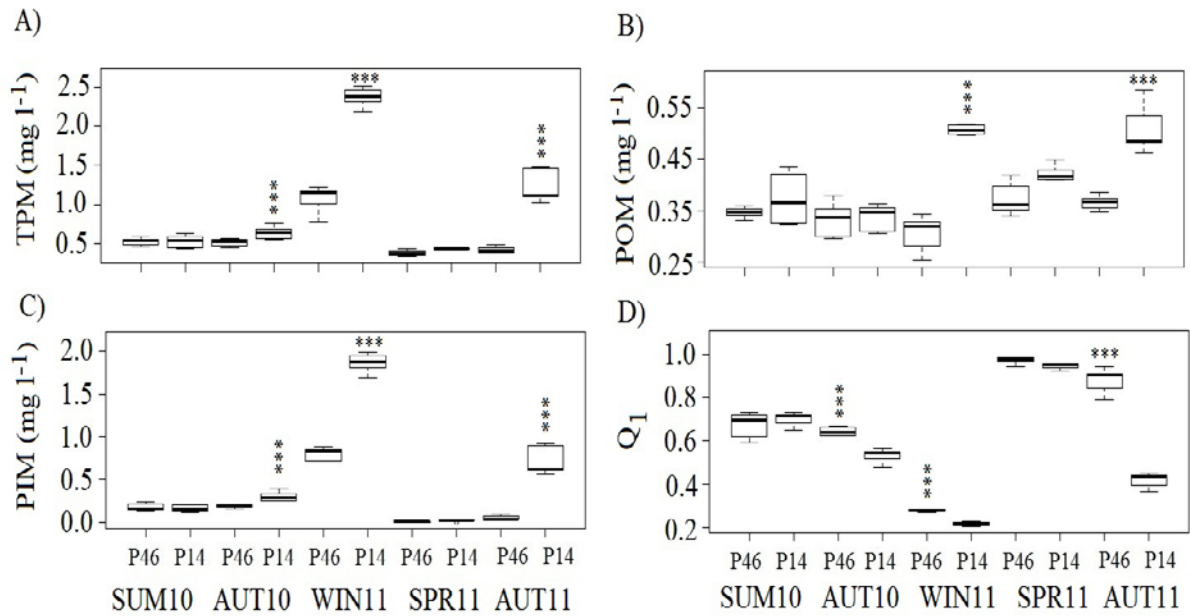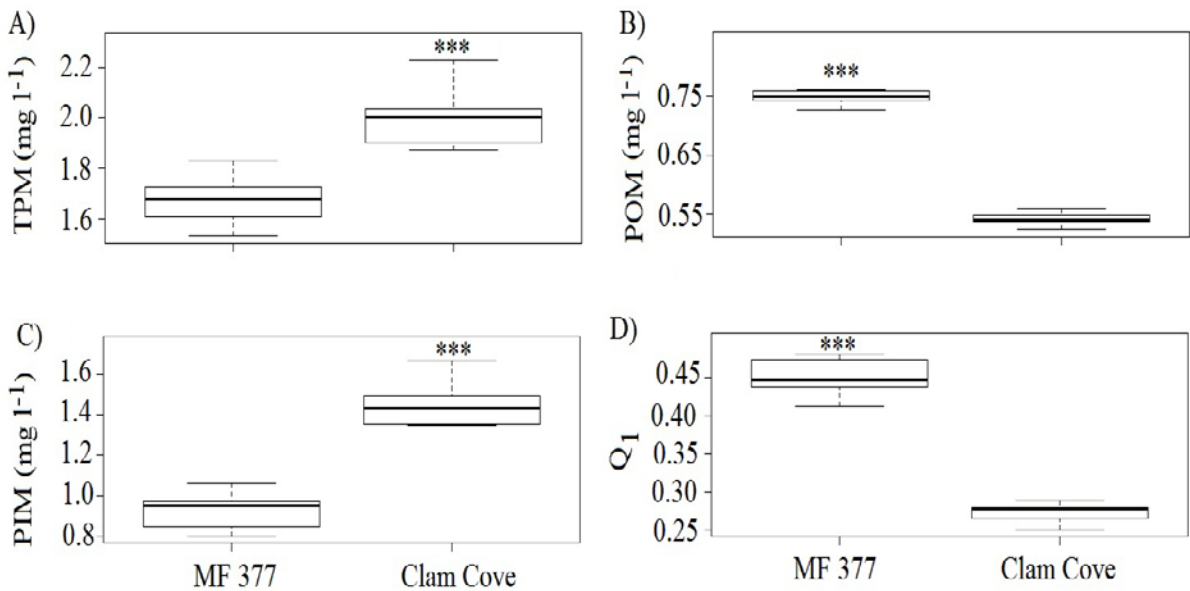


Fig. 3. Box- plots representing the mean summer values of A) total particulate matter (TPM; mg l $^{-1}$); B) particulate organic matter (POM; mg l $^{-1}$); C) particulate inorganic matter (PIM; mg l $^{-1}$); D) $Q_1$ (POM / TPM) registered at the long-line MF 377 and the IMTA site at Clam Cove in the Bay of Fundy. Significant differences are denoted by $P < 0.001$***.

The average seston organic content measured over the spring-summer was 82 and 82.5 % at the rafts distant (n= 31) and close (n = 29) to the cages, respectively, while during the autumn-winter period values at rafts P-46 and P-14 were 60 and 38.3 %. The results of the two-way ANOVA indicated that the sampling site, season and the interaction term (site × season) had a significant effect on the TPM, POM, PIM and $Q_1$ ($P < 0.001$, Table 2). The *post-hoc* analyses showed significantly higher levels of TPM, POM and PIM fractions by almost two-fold at the raft close to the fish cages compared with the other raft during autumn and winter (Tukey HSD, $P < 0.001$), but no differences for POM levels during autumn 2010 (Fig. 2 A, B, C). On the other hand, $Q_1$ showed significantly lower values during autumn and winter at the raft close to the fish cages than at P-46 (Fig. 2 D).

The mean seston levels and SD registered during the summer period at the Bay of Fundy are shown in Figure 3. TPM at the mussel monoculture site MF 377 (n= 9) ranged from 1.53 to 1.83 mg l$^{-1}$, while TPM at the Clam Cove IMTA location (n = 9) ranged from 1.87-2.23 mg l$^{-1}$ (see Table 1). Average TPM values were $1.68 \pm 0.10$ mg l$^{-1}$ and $1.99 \pm 0.11$ mg l$^{-1}$ for MF 377 and Clam Cove, respectively. Levels of POM and PIM computed at MF 377 (n = 9) ranged from 0.72-0.82 and 0.80-1.06 mg l$^{-1}$, while values at the IMTA site (n = 9) ranged from 0.52-0.57 and 1.34-1.66, respectively. Values of $Q_1$ observed at MF 377 varied from 0.41 to 0.48 while values obtained at Clam Cove ranged from 0.35 to 0.28 (Table 1). The one-way ANOVA showed a significant effect of the sampling site on the TPM, organic and inorganic fractions and $Q_1$ ($P < 0.001$, Table 2). TPM and PIM were significantly greater at the Clam Cove IMTA site than at the long-line MF 377 (Fig. 3 A, C), while POM and $Q_1$ were significantly lower at Clam Cove (Fig. 3 B, D) than at MF 377 (Tukey HSD, $P < 0.001$).

|  | Site | | | | Season | | | | Site x season | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | SS | MS | F | P | SS | MS | F | P | SS | MS | F | P |
| **TPM** | | | | | | | | | | | | |
| Lorbé | 2814.36 | 2814.36 | 84.59 | < 0.001*** | 11325.21 | 2831.30 | 85.10 | < 0.001*** | 2395.48 | 598.87 | 18 | < 0.001*** |
| Fundy | 0.44 | 0.44 | 39.29 | < 0.001*** | | | | | | | | |
| **POM** | | | | | | | | | | | | |
| Lorbé | 5336.57 | 5336.57 | 58.25 | < 0.001*** | 4579.46 | 1144.86 | 12.5 | < 0.001*** | 3844.68 | 961.17 | 10.49 | < 0.001*** |
| Fundy | 0.20 | 0.20 | 407.31 | < 0.001*** | | | | | | | | |
| **PIM** | | | | | | | | | | | | |
| Lorbé | 1797.66 | 1797.66 | 102.80 | < 0.001*** | 13298.15 | 3324.54 | 190.1 | < 0.001*** | 2242.24 | 560.56 | 32.05 | < 0.001*** |
| Fundy | 1.23 | 1.23 | 134.31 | < 0.001*** | | | | | | | | |
| **$Q_1$** | | | | | | | | | | | | |
| Lorbé | 1262.39 | 1262.39 | 92.64 | < 0.001*** | 14253.84 | 3563.46 | 261.5 | < 0.001*** | 1938.32 | 484.58 | 35.56 | < 0.001*** |
| Fundy | 0.14 | 0.14 | 430.97 | < 0.001*** | | | | | | | | |

Table 2. Results of the two-way and one-way ANOVA testing the influence of site, season and the interaction term site x season on total particulate matter (TPM), organic (POM) and inorganic (PIM) fractions of seston and the quality index $Q_1$ in Lorbé raft polygon and the Bay of Fundy. Significant differences are denoted by *** $P < 0.001$.

### 3.2. Mussels absorption efficiency (AE)

Values for the absorption efficiency of *M. galloprovincialis* at the two Lorbé rafts varied seasonally, with the highest values observed during the spring-summer period and lowest values during the autumn-winter period (Fig. 4). Mean AE values were highest at both rafts during the spring survey (97.5 % at P-46 and 94.6 % at P-14) and lowest during winter (54.8 and 34.9 % at P-46 and P-14, respectively) (Table 1). The mean annual AE for mussels from raft P-46 (n = 34) was 79.8 ± 15.3 %, while the AE at P-14 (n = 39) averaged 67.7 ± 19.7 %. The absorption efficiency of the mussels from the raft distant from the fish cages (P-46) ranged from 54.8 - 97.5 %, while the AE of the mussels cultured at the raft next to the cages (P-14) ranged from 34.9 - 94.6 % (Table 1).

Results of two-way ANOVA showed that there was no site or seasonal effects *per se*, as the significant effects detected for the site on the AE depended on the sampling time ($P < 0.001$, Table 3). Thus, the *post-hoc* test indicated that the values of absorption efficiency were significantly higher in mussels cultured at P-46 than in mussels reared close to the fish cages during autumn and winter (Tukey HSD, $P < 0.001$). Tukey's pairwise comparisons showed no discernible differences in the AE of mussels cultured at P-46 during the spring and summer surveys in relation with P-14.
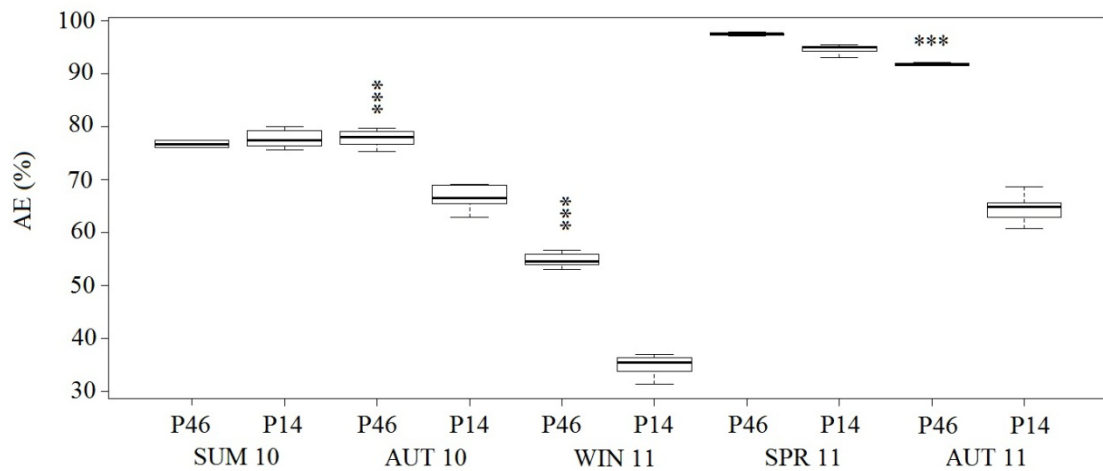


Fig. 4. Box-plots showing the mean seasonal values of absorption efficiency (AE; %) of the mussel *Mytilus galloprovincialis* measured at the rafts distant (P-46) and close (P-14) to the fish cages. Significant differences are denoted by $P < 0.001$***.

|  | **Site** | | | | **Season** | | | | **Site x season** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | SS | MS | F | P | SS | MS | F | P | SS | MS | F | P |
| **AE** | | | | | | | | | | | | |
| Lorbé | 2870 | 2870 | 152.91 | < 0.001*** | 23469 | 5867 | 313 | < 0.001*** | 2337 | 584 | 31.12 | < 0.001*** |
| Fundy | 875.93 | 875.93 | 87.293 | < 0.001*** | | | | | | | | |

Table 3. Results of the two-way and one-way ANOVA testing the influence of site, season and the interaction term site x season on mussel *Mytilus galloprovincialis* absorption efficiency in Lorbé and the Bay of Fundy. Significant differences are denoted by *** $P < 0.001$.

A positive and significant relationship was obtained between the AE and the food quality index $Q_1$ for *M. galloprovincialis* in the raft distant (r = 0.96, *P* < 0.001) and close (r = 0.93, *P* < 0.001) to the fish cages (Table 4). This significant correlation was confirmed by the backwards multiple step regressions that showed how the highest variability for the AE was best explained by $Q_1$ at the raft distant (F = 2455.7, n= 34, $R^2$ = 0.98, *P* < 0.001) and close (F = 586.3, n= 39, $R^2$ = 0.94, *P* < 0.001) to the fish cages (Table 5). The AE was plotted against the inverse transformation of the food quality and the best model was established by means of simple linear regression (Fig. 6). The inverse transformation of the independent variable was performed to linearize the hyperbolic trend previously observed for the relationship between both variables (Navarro et al., 1996; Hawkins et al., 1996; Cranford and Hill 1999). The following linear models explained 89 and 93 % of the variance for P-46 (Eq. 1) and P-14 (Eq. 2), respectively.

*P-46 AE = -15.65 · 1/$Q_1$ + 107.12*                                                    Eq. 2

*P-14 AE = -14.89 · 1/$Q_1$ + 101.56*                                                    Eq. 3

The analysis of covariance (ANCOVA) of the models showed no significant differences in elevations obtained for the rafts P-46 and P-14 (ANCOVA, *P* > 0.001). The AE decreased as a function of decreasing quality of seston and the model predicted AE = 0 when the percentage of organic content reached levels below 14 % for mussels at both rafts.

|   | Site | TPM | POM | PIM | $Q_1$ |
|---|---|---|---|---|---|
| AE | P-46 | -0.94*** | 0.96*** | -0.95*** | 0.96*** |
| (%) | P-14 | -0.94*** | -0.60*** | -0.95*** | 0.93*** |

Table 4. Pearson's correlation coefficients for the relationship between the absorption efficiency (AE; %) and the environmental variables measured at raft P-46 and P-14 in Lorbé raft polygon. Significant levels were denoted as *$P$ < 0.05, ** $P$ < 0.01, *** $P$ < 0.001.

|          | B      | SE of B | t      | F        | $R^2$     |
|----------|--------|---------|--------|----------|-----------|
| AE P-46  |        |         |        |          |           |
| Intercept| 36.990 | 0.908   | 40.729 |          |           |
| $Q_1$    | 62.189 | 1.254   | 49.555 | 2455.700 | 0.987***  |

$R^2$= 0.987, Adjusted $R^2$= 0.986, n=34, F(1,32)=2455.8; $P < 0.001$

| AE P-14  |        |         |        |          |           |
|----------|--------|---------|--------|----------|-----------|
| Intercept| 24.681 | 3.107   | 7.944  |          |           |
| $Q_1$    | 60.522 | 1.883   | 32.146 | 586.338  | 0.940***  |

$R^2$= 0.988, Adjusted $R^2$= 0.987, n=39, F(2,36)=1502.3; $P < 0.001$

Table 5. Summary of the backwise multiple linear regressions models obtained for the absorption efficiency of mussels at Lorbé raft polygon and the variance explained using $Q_1$ as the independent variable.

Mean AE values obtained for blue mussel *M. edulis* at MF 377 (n = 9) were 69.4 ± 3.6 %, while AE levels at Clam Cove (n = 9) were 55.6 ± 2.9 % (Fig. 5). Values observed for mussels cultured in the long-line MF 377 ranged from 63.8 to 74.2 %, while values at Clam Cove IMTA site ranged from 51.4 to 59.2 %. One-way ANOVA showed a significant effect of the site on the AE of blue mussel (Table 3, $P < 0.001$). The *post-hoc* testing highlighted significantly higher AE values for mussels cultured at the mono-culture site than at the IMTA site (Tukey HSD, $P < 0.001$).
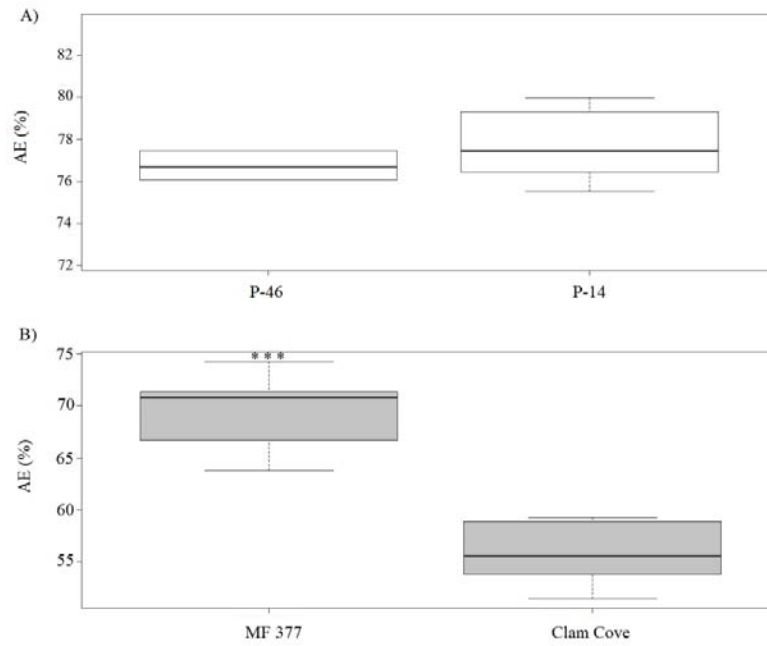
Fig. 5. Box-plots showing the mean summer values obtained for the the absorption efficiency (AE; %) of the mussel *Mytilus galloprovincialis* (A) and *Mytilus edulis* (B) measured at the Lorbé raft polygon (Galicia, NW Spain) and in the Bay of Fundy (Canada). Significant differences are denoted by $P < 0.001***$.
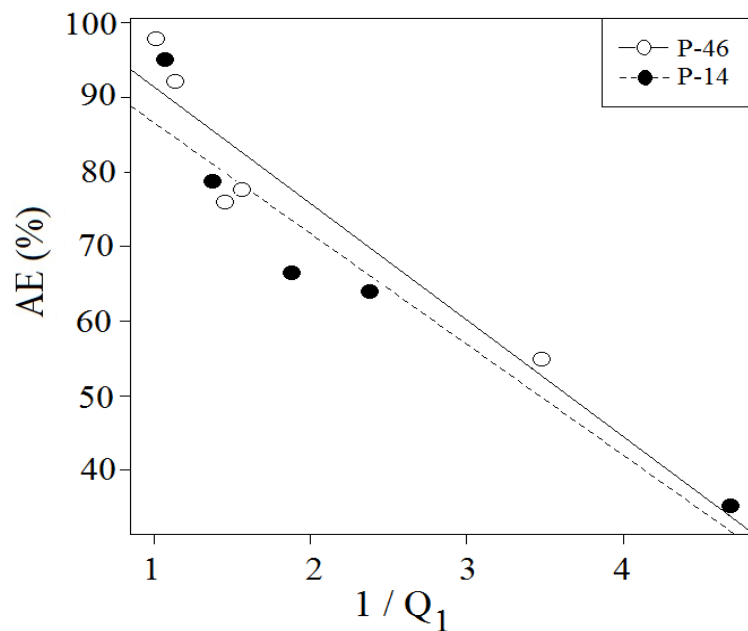


Fig. 6. Linear regression established between the absorption efficiency (AE; %) and the food quality index $Q_1$ for the rafts P-46 (full line with hollow circles) and P-14 (dashed line with solid circles).

# 4. Discussion

## 4.1. Environmental parameters

The seston concentrations recorded in Lorbé were similar to average values reported for the Galician Rías ($< 3$ mg l$^{-1}$) which are considered as low seston environments (Figueiras et al., 2002; Duarte et al., 2008; 2012) where mussel productivity relies on the large fraction of phytoplankton comprising the seston. Due to their filter-feeding habits, mussels are subjected to large natural fluctuations in seston quality that occur at long and short-term temporal scales. Long-term fluctuations include seasonal changes in primary production induced by upwelling (March-October) and downwelling (October-February) (Figueiras et al., 2002). Maximum seston quality found in May was likely explained by the spring phytoplankton bloom that traditionally occurs in the Rías during this period, while minimum values registered in October and February corresponded with the downwelling season. Inter-annual variability between seston parameters measured in autumn of 2010 and 2011 was limited to differences in POM levels, which were higher in October 2011 than 2010. Short-term variations in food quality detected in the present study suggested the occurrence of coastal resuspension events. Silt and clay resuspension from the benthos results in an increase in suspended particulate inorganic matter and reduced food quality. In this study, resuspension was demonstrated by the high levels of TPM recorded during autumn and winter at the raft next to the cages that were matched by significantly high concentrations of inorganic particles. Resuspension of particulate inorganic mater contained in Lorbé' sediments increased the inorganic content of seston by a factor of two, resulting in a decrease of food quality available for shellfish (Fig. 2 D). Zuñiga et al. (2014) showed that stormy conditions during the winter resulted in higher current speeds at the raft close to the fish cages (13.1 cm s$^{-1}$) than at P-46 (7.8 cm s$^{-1}$) and an increase in the supply of inorganic material by continental runoff. Rivers Eume and Mandeo runoff was 100-200 m$^3$ s$^{-1}$ during October 2010-February 2011 and $< 50$ m$^3$ s$^{-1}$ in July 2010-May 2011 (Zuñiga et al., 2014). The fact that the raft adjacent to the fish cages had higher current speeds and was closer to the input of sediments from the coastal runoff may explain why it was more affected

by sediment resuspension than the other culture unit (Fig. 1). Cheshuk et al. (2003) found no significant differences in particle concentration between the fish-pen and control sites. However, they did detect slightly higher particle concentrations at the IMTA farm (1 % increase above ambient POM levels during the peak period of salmon biomass) throughout the different salmon production stages that they attributed to the sedimentation and resuspension of fish faeces and pellets beneath the net-pens (Cheshuk et al., 2003) as opposed to any seasonal effects. In the present study, no differences in seston quantity and quality were detected among the two Lorbé rafts during the spring and summer surveys, indicating that the close proximity of the mussel raft to the fish cages had no significant effect on their particulate food supply. Similarly, in the study of Gao et al. (2006), sediment resuspension from the seabed was only detected at the control site during a winter monsoon. The mussels-fish IMTA site had lower levels of PIM, likely owing to the relatively weaker currents.

The range of TPM values measured at the two Bay of Fundy sites (mussel monoculture and mussel-salmon IMTA) were similar to those previously measured during the summer (June) at three IMTA mussel-salmon sites in the Bay of Fundy (MacDonald et al. 2011). In contrast to the results of the present study, they found that TPM was significantly higher adjacent to salmon cages than at the reference site (1.79-4.39 mg l$^{-1}$). Significantly greater TPM and PIM levels measured in the present study at the Clam Cove IMTA site, compared to the mussel site, corresponded with lower POM and seston quality. The Passamaquoddy region in the Bay of Fundy has a very large tidal range ($\geq$ 6 - 8 m) and high tidal currents velocities due to a combination of the semidiurnal tide of the North Atlantic Ocean with the topography of the Bay of Fundy system (Gregory et al., 1993; Wildish et al., 1993). Thus, tidal resuspension events may explain spatial differences in the quality of seston between our two sites. Work by Reid et al., (2010) at a mussel - salmon culture site in the Bay of Fundy showed that the quality of the seston decreased with increasing TPM, suggesting periods of inorganic sediment resuspension.

Differences found in seston quality in Lorbé and the Bay of Fundy suggested that the proximity to the fish cages did not enhance the organic content present in the diet for the bivalves. However, it is necessary to highlight that these results contrast with those from other studies

where higher levels of TPM and POM were found next to fish cages and which were attributed to fish waste effluents (Jones and Iwama, 1991; Stirling and Okumus, 1995, MacDonald et al., 2011). The amount of organic particles found close to the cages could differ between IMTA sites depending on the location of cage and reference sites relative to poorly known waste transport processes around fish cages, site-specific particle advection and dilution dynamics forced by local hydrodynamic conditions, temporal and spatial variations in waste production related to local fish husbandry practices (e.g. biomass, feeding amount and timing), and natural patchiness in the seston (e.g. Troell and Nordberg, 1998; Cheshuk et al., 2003; Troell et al., 2009). Thus, additional information on spatial and temporal variations of the fish nutrient plume, seston particle dynamics, current speed and direction are needed before a final conclusion on IMTA feasibility is achieved.

## 4.2. Absorption efficiency

The absorption efficiency values measured at the Lorbé rafts are within the range described for *M. galloprovincialis* cultured in the Galician Rías, with maximum and minimum values being coupled with the upwelling-downwelling cycle. Previous studies estimated AEs of 21-75 % in rafts located in different sites of the Ría de Arousa (Navarro et al., 1991), 80 % for mussels cultured in the same Ría (Pérez-Camacho et al., 2000) and values ranging from 55-73 % in spring-summer and 26-71 % in autumn-winter (Babarro et al., 2003). The AE values obtained in the experiment of the Bay of Fundy were also within the wide range reported for *M. edulis* feeding on natural seston of 0-90 % (Widdows and Bayne, 1971; Bayne and Widdows, 1978; Cranford and Hill, 1999). The seasonal pattern obtained for the AE of mussels in Lorbé corresponded with the variations observed for the food quality ($Q_1$). The reduction of the food quality during the autumn-winter resuspension events resulted in absorption values being 26 % lower at the raft near the fish cages. On the other hand, an enhancement in the seston quality during spring and summer increased AE to similar levels at both mussel rafts. Mussels from the two sites in the Bay of Fundy displayed a similar pattern, and the absorptive behaviour was 20

% greater in the site distant from the salmon farm, where seston quality was higher. This finding agrees with previous studies that observed how the AE of *M. galloprovincialis* and *M. edulis* increased with greater food quality (Navarro et al., 1991; 1996; Hawkins et al., 1996; Cranford and Hill 1999; Perez-Camacho et al., 2000; Babarro et al., 2003; Reid et al., 2010). This association is expected given that AE calculations are based partially on the diet organic content (Eq. 1). However, the nature of this relationship varies between bivalve species and the degree of dietary adaptation (Cranford and Grant, 1990; Cranford, 1995; Cranford and Hill, 1999). Navarro et al. (1991) modeled the AE of *M. galloprovincialis* as an exponential function of food quality and graphically demonstrated how the AE increased with Q (organic content per unit volume), obtaining an AE = 0 when Q was 24%. The logarithmic model proposed by Reid et al. (2010) suggested an increase in the AE of *M. edulis* with larger organic content (OC) of different laboratory diets and a field diet consisting of a mixture of salmon effluents and natural seston.

The conditions in our study were comparable to the field experiment of Reid et al. (2010), that reported an AE = 54 % due to the low OC = 36 % contained in the salmon effluents. Similarly, co-cultured mussels in Clam Cove and bivalves from a raft close to sea bream cages in Lorbé presented AEs of 55 and 67 % with a diet organic content of 27 and 54 %, respectively. The highest levels of AE (90 %) obtained by Reid with a laboratory diet of salmon feed were consistent with the high organic content (OC = 93 %). However, when a spat formula (OC = 77 %), salmon faeces (OC = 77 %) and a diatom diet (OC = 66 %) were tested, the AE was reduced to 87, 86 and 81 % respectively. Similar results were obtained by Lefebvre et al. (2000) for Pacific oyster (*Crassostrea gigas*) exposed to fish effluents in laboratory conditions. The AE was lower (56 %) when exposed to fish effluents than a diatom diet (66-70 %), although values were considered high for a diet based on detritus with low organic content.  In addition, MacDonald et al. (2011) observed no differences between the AE of *Mytilus edulis* exposed to similar concentrations of the microalgae *Isochrysis galbana* and fish food pellets in the laboratory, obtaining an AE of 40 % for both diets. However, in a second experiment comparing the exhalent siphon area (ESA) between mussels' culture close and distant to salmon net-pens in

the Bay of Fundy, MacDonald et al. (2011) recorded significantly higher ESAs at the salmon farm. The authors obtained a correlation between the ESA and the high levels of food quantity (measured by TPM) and quality (measured by POM and energy content of the seston) detected in the salmon farm.

The results of this field experiment emphasized that the AEs of mussels cultured near fish cages with low (Lorbé) or high (Bay of Fundy) stocking density were not significantly greater than the AEs of mussels reared distant from the cages. Although laboratory and field studies have demonstrated the capacity of mussels to utilize fish pellets and faeces we can't confirm that mussels in this study were absorbing organic particles of fish origin, as levels of seston detected close to the cages were not above the levels registered at the sites distant from the cages. The variability of the results found for different mussel-fish IMTA farms can be explained by a combination of several factors. Firstly, different mussel species, such as *M. edulis* (MacDonald et al., 2011) and *M. planulatus* (Cheshuk et al., 2003), may differ in their capacity to absorb feed fines. Secondly, commercial farming and husbandry practices (structures, standing stock, etc) differ greatly between sites and some culture practices can affect the spatial distribution of suspended particulate matter. Sampling depths, timing and distance of sampling sites from the fish cages also vary markedly between study sites. Cheshuk et al. (2003) recommended to culture mussels close to the fish cages (within 50 m) on several sides of the cages and deeper than 5 m. However, we emphasize that additional information on the supply and utilization of organic wastes from fish culture is needed to determine optimal IMTA designs.


## 5. Conclusions

This study found no evidence of increased seston concentrations (food quantity) or organic content (food quality) at commercial mussel aquaculture sites located near the two fish cage sites in Spain and Canada. The results suggested that mussels *M. edulis* and *M. galloprovincialis* cultured close to the fish cages did not exhibit greater AE compared with the bivalves cultured distant from the net-pens. Differences in the AE of mussels held close and distant to the fish

cages can be explained by natural spatial and temporal differences in seston quality. Coastal resuspension dynamics were the most likely explanation for the site differences detected and these short-term changes in particulate inorganic matter are likely to affect any coastal area. Near-shore particle gradients were independent of the presence-absence of fish pens and decreased the filter-feeder's absorption efficiency. Thus, the success of the integrated culture would, in part, be conditioned by the food quality available for the filter-feeders. Assessments of temporal and spatial variations in culture density, hydrodynamic characteristics of the selected area and variability in the quantity and quality of seston could be of great importance for evaluating the implementation of IMTA systems.

# Chapter 3

## Effects of seasonal variations in phytoplankton on the bioenergetic responses of mussels (*Mytilus galloprovincialis*) held on a raft in the proximity of red sea bream (*Pagellus bogaraveo*) net-pens

# ABSTRACT

The seasonal variability of the physiological components of the Scope for Growth (SFG) of mussels *Mytilus galloprovincialis* was investigated in a raft adjacent (170 m) to fish net-pens and compared with a raft 550 m distant from the cages in Ría Ares-Betanzos (Galicia, Spain). Chlorophyll and phytoplankton size-classes were determined in the field, simultaneously with SFG. Average chlorophyll-*a* was $0.65 \pm 0.24$ µg l$^{-1}$, while nanophytoplankton (2-20 µm) was the most abundant size-class, ranging from 50 to 70% of the total chlorophyll. The temporal pattern found for chlorophyll-*a* and phytoplankton size-classes reflected the upwelling-downwelling events and were correlated with the feeding, digestive and metabolic rates. Nanophytoplankton and microphytoplankton were preferentially cleared and ingested by mussels. There were no significant differences between the chlorophyll and phytoplankton size-classes among rafts. The lack of any enhancement in food availability resulted in no significant increase in the SFG of mussels beside the fish cages. Maximum SFG corresponded with the autumn ($16.60 \pm 7.90$ J h$^{-1}$) and spring ($12.72 \pm 9.32$ J h$^{-1}$) chlorophyll maximums. An abnormally hot summer and reduced chlorophyll levels resulted in lower energy intake, significantly higher metabolic expenditure and a negative SFG ($- 34.57 \pm 12.55$ J h$^{-1}$). Any particulate wastes and potential fish-derived chlorophyll enhancement would be rapidly diluted by the currents, while the placement of bivalves too distant from the fish farm in an environment with high supplies of natural seston may explain the lack of an augmented SFG of the co-cultured mussels.

***Key words:*** *mussels; fish waste; physiological energetics; Scope for Growth; phytoplankton size-classes; chlorophyll-a.*

# 1. Introduction

Since the 1980s the rapid population growth coupled with a rising seafood demand has led aquaculture to become the fastest growing food sector in the world. Shellfish farming is one of the most important mariculture products and represents 42.8 % of the global production (FAO 2009). The Galician Rías (N.W. Spain) have a thriving mussel industry. Galicia is the third largest producer of mussels (*Mytilus galloprovincialis*) in the world, with 3,300 floating rafts that produce 250,000 tons year $^{-1}$ worth more than \$165 million US (Labarta et al., 2004). Mussel farming started in 1946 and provides 9,000 and 20,000 direct and indirect jobs (Labarta et al., 2004). The high production of phytoplankton during the upwelling season (March-October) provides food of high quality (50% organic content) that is efficiently absorbed (60%) by the cultured mussels (Figueiras et al., 2002). The sheltered coasts of the Galician Rías also provide a suitable environment for open-water intensive sea cage fish-farming, but environmental concerns and limited space to allocate the cages are the main issues restraining the expansion of this activity. Caged fish-farming releases large amounts of solid organic nutrients (i.e. organic C, N and P contained in undigested feed pellets and feces) and dissolved inorganic nutrients (i.e. $NH^{3+}$, $PO_4^{3-}$ and $CO_2$ through excretion and respiration) (Wang et al., 2012) that have been associated with phytoplankton blooms near the fish cages (Pitta et al., 2009; Sarà et al., 2012). Several studies have indicated that mussels could be cultured alongside fish cages to utilize the additional phytoplankton production, the unconsumed feed and fish feces as an additional food source, while simultaneously offering environmental, economical and social benefits (MacDonald et al., 2011; Lander et al., 2012; Handå et al., 2012a; 2012b). The synergistic culture of finfish (fed aquaculture) in close proximity to mussels or other organic (e.g. sea cucumbers) or inorganic (e.g. seaweed) extractive species, is a practice known as Integrated Multi-Trophic Aquaculture (IMTA) (Chopin et al., 2008; 2012). IMTA is considered a potential strategy to recycle surplus organic and inorganic nutrients released from fish farms and simultaneously increase the growth of the extractive species.

Most investigations assessing mussels' ability to uptake fish unconsumed feed and feces have measured the growth (Stirling and Okumus, 1995; Lander et al., 2012), the fatty acid (Handå et al., 2012a; b) and isotopic profile of the mussels (Gao et al., 2006; Redmond et al., 2010). Conversely, very little is known about the physiological energetics of bivalves cultured in the proximity to fish cages, since previous studies have focused mainly on the absorption efficiency of the fish particulate surplus (Reid et al., 2010; MacDonald et al., 2011; Irisarri et al., 2013). Measurements of the different physiological rates of a bivalve (clearance, ingestion, absorption, respiration, excretion) can be integrated to determine the net energy balance (difference between the energy absorbed from the ingested food and the energy lost in respiration and excretion), which is commonly referred to as the "Scope for Growth" (SFG) (Winberg, 1960). SFG variations reflect spatio-temporal fluctuations in environmental conditions (Albentosa et al., 2012). To our knowledge, no previous studies have investigated the SFG of bivalve species cultured in proximity to fish cages either in the field or under laboratory conditions. However, SFG is one of the main approaches to model bivalve growth and has been successfully used in a range of different mytilid species exposed to varying environmental conditions. Hence, several studies have measured the SFG of the mussel *M. galloprovincialis* (Navarro et al., 1991; 1996; Pérez-Camacho et al., 2000; Helson and Gardner, 2007; Sarà and Pusceddu, 2008; Albentosa et al., 2012; Fernández-Reiriz et al., 2012).

Previous studies on the utilization of fish effluents by mussels have been performed with a limited number of individuals, which does not allow for a general conclusion on the potential contribution of commercial-scale mussel farming mitigating fish nutrient impacts (Handå et al., 2012a). In this study, this issue was overcome by selecting a commercial mussel raft operating in the proximity of a fish farm of red sea bream in the Ría Ares-Betanzos (Galicia, NW Spain).

This study investigated the seasonal variability in energy uptake and utilization by the edible mussel *M. galloprovincialis*. The primary objective was to determine if any particulate food enhancement from fish wastes increased the SFG of the commercial mussels compared to mussels contained on a reference raft distant from the fish farm. Measurements of clearance, ingestion, absorption respiration and excretion rates were determined in the field under natural

conditions of food availability and were integrated to determine O:N ratio, SFG and net growth efficiency. A second objective was to analyze temporal and spatial variations in total phytoplankton biomass (chlorophyll-*a*) and phytoplankton size-classes, to investigate: (1) their importance in the mussels' diet, (2) the physiological responses of mussels to natural dietary fluctuations and (3) to determine if any dissolved inorganic nutrients contained in fish waste enhanced the local phytoplankton biomass.

## 2. Material and methods

### 2.1. Study site

Field studies were carried out in the Lorbé raft polygon in the Ría Ares–Betanzos, NW Spain (Fig.1; Latitude 43°23'24.74''N; Longitude 8°17'48.30''W). All mussels *M. galloprovincialis* used in this study had the same origin and were cultured on 12 m long ropes at a stocking density of 700-1000 mussels $m^{-1}$. Water sampling and physiological measurements were conducted at two commercial rafts. Raft P-14 (43º 23.3328' N; 8º 17.2878' W) was the culture unit closest to the fish cages in the Lorbé raft polygon (170 m north of a red sea bream farm), while raft P-46 (43º 23.4876' N; 8º 17.109' W) was used as a reference station and was situated 550 m north from the net-pens. Raft P-14 had an average annual population of 3,856 x $10^3$ mussels, while raft P-46 had an average population of 4,299 x $10^3$ mussels (Table 1).

The fish farm of red sea bream (*Pagellus bogaraveo*) consisted of 48 net-pens, with an extra 2 empty cages. Each pen is 28.5 m in diameter, 6 m in depth and an approximate volume of 3,692.64 $m^3$ (Guisado et al., 2007). The fish farm has an estimated annual stocked biomass of 450 tons, and the estimated stocked biomass during the sampling period was around 70 tons, with an approximate culture density of 0.4 kg $m^{-3}$. Fish were fed *ad libitum*, with a constant daily feeding regime representing 0.5-0.7 % of their fresh body weight (Guisado et al., 2007). Fish were hand-fed a commercial diet of heat extruded pellets (Skretting B4 power 2 P). During the course of this study the fish farm was stocked with more than a single cohort of fish, implying that there were no major seasonal variations in feed use.

The general pattern of water circulation in the Ría Ares-Betanzos consists of oceanic water from the continental shelf entering along the southern margin of the Ría, from where it moves towards the east, then southwards, north-easterly and finally westward into the Atlantic (Sánchez-Mata et al., 1999). The Ría has a prevalent positive circulation scheme with a two-layered residual circulation pattern, this means that the upper layer moves seaward, while denser and deeper layers of oceanic water move landward (Sánchez-Mata et al., 1999 and references therein). Predominant north-easterly winds during spring and summer usually enhance this positive circulation pattern, whereas south-westerly winds blowing during autumn and winter can reverse the positive estuarine circulation (Bode and Varela, 1998).

The Ría has a semi-diurnal tidal frequency, with spring and neap mean tidal ranges of 4.14 and 0.02 m, respectively (Sánchez-Mata et al., 1999). Tidal currents are more important than wind-induced or wave-induced currents in the Ría Ares-Betanzos (Sánchez-Mata et al., 1999) and maximum tidal current speed in the middle of the Ría at 3 m depth is 2.2 cm s$^{-1}$ (Piedracoba et al., 2014). Tidal currents are rectilinear, and accommodate to the shape of the Ría, flowing with a mean along-channel orientation in an anticlockwise angle of 139 ° (i.e. 0° starts on the east) (Piedracoba et al., 2014; Fig. 1C). Tidal currents explain 53.4 % of the total variance of surface currents in Lorbé raft polygon and have an average speed of 1.7 cm s$^{-1}$ at 1 m depth (Piebracoba et al., 2014). Average residual current speeds at P-14 and P-46 are 3.70 and 3.64 cm s$^{-1}$, respectively (Zuñiga et al., 2014). During the sampling period, maximum total current speeds at the raft next to the cages ranged from 5.1-13.1 cm s$^{-1}$, whereas maximum total current speed at the reference raft ranged from 5.3-11.5 cm s$^{-1}$. During the ebb tide water flows from the fish cages to the mussel rafts, whereas during the flood tide it flows from the rafts towards the cages (Fig. 1). Hence, mussels might potentially be within the transport pathway of fish particulate wastes during a large percentage of the tidal cycle.

All measurements were conducted within two consecutive days during five seasonal campaigns. The campaigns were selected to represent typical oceanographic scenarios of the Rías: 1) summer upwelling (6th and 7th July 2010), 2) autumn bloom (5th and 6th October 2010 and 24th and 25th October 2011), 3) winter mixing (7th and 8th February 2011) and 4) spring bloom (2nd

and 3rd May 2011). The two sampling campaigns in October were executed to test for inter-annual variance. Physiological rates and environmental parameters were determined simultaneously in the field, onboard a boat moored to each raft to maintain ambient conditions of temperature, salinity, and food availability.
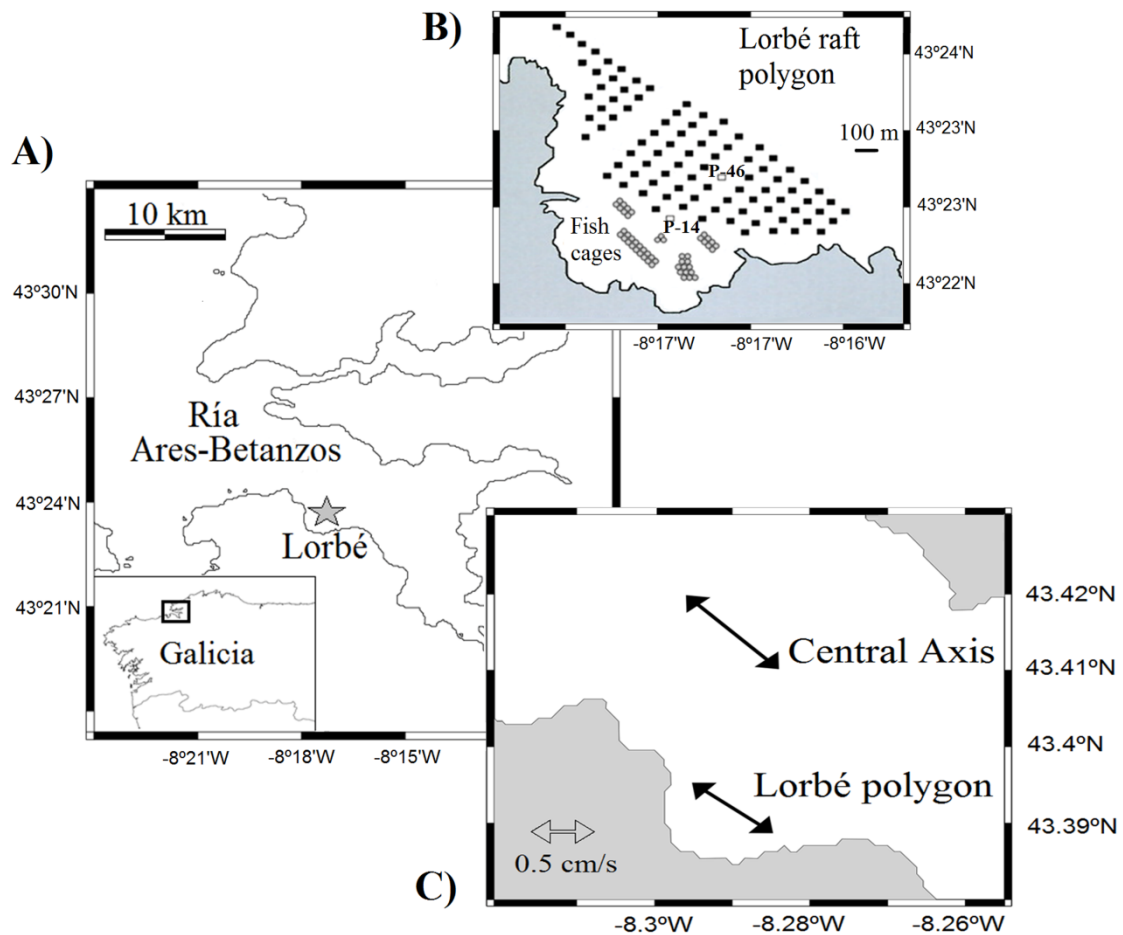


Fig. 1. Map indicating: A) the location of Lorbé raft polygon in the Ría Ares-Betanzos (Galicia, N.W. Spain); B) the position of the fish cages (circles) and the rafts in the polygon (squares), with white squares for raft P-14 (at 150 m from the middle of the fish farm) and P-46 (550 m from the middle of the fish cages); C) tidal vectors that show the speed (cm s^{-1}) and orientation of tidal currents at Lorbé polygon (1 m depth) and at the central axis of the Ría (3 m depth).

Percents of total variance explained by the tide were 53.4% and 51.5%, respectively (modified from Piedracoba et al., 2014).

| Season | Mussels per raft | |
|---|---|---|
| | P-46 | P-14 |
| Summer 2010 | $6,000 \times 10^3$ | $4,687 \times 10^3$ |
| Autumn 2010 | $4,819 \times 10^3$ | $4,477 \times 10^3$ |
| Winter 2011 | $4,216 \times 10^3$ | $1,440 \times 10^3$ |
| Spring 2011 | $2,175 \times 10^3$ | $3,968 \times 10^3$ |
| Autumn 2011 | $4,286 \times 10^3$ | $4,710 \times 10^3$ |
| Average | $4,299 \times 10^3$ | $3,856 \times 10^3$ |

Table 1. Mean seasonal mussel population at the reference raft P-46 (further from the net-pens) and P-14 (adjacent to the fish farm).

## 2.2. Chlorophyll-a and phytoplankton size-classes determination

Seawater from 3 m depth within each raft was supplied by a peristaltic pump. Three replicates of 1 l of seawater (n = 30) were collected from the outflow of an empty chamber used as control during the physiological experiments. Seawater collected from each site and sampling date was filtered through a serial polycarbonate filtration unit for determination of chlorophyll-*a* concentrations within three phytoplankton size-classes according to their equivalent spherical diameter (ESD). Phytoplankton were fractionated into picophytoplankton (0.2-2 µm ESD), nanophytoplankton (2-20 µm ESD) and microphytoplankton (> 20-50 µm ESD) size-classes and the total chlorophyll-*a* (chl-*a*, µg $l^{-1}$) was calculated as the sum of the chlorophyll determined in each of the size classes. All filters were frozen at – 20 °C to facilitate cellular lysis and enhance chlorophyll extraction. Pigments were extracted using 5 ml of 90 % acetone as a solvent, and left in the dark for 12 h. The solution was then centrifuged at 4500 rpm at 10°C for 10 min to isolate the chlorophyll extract from the filter residues. Chlorophyll was quantified

using a Perkin-Elmer Lambda 35 UV/VIS spectrophotometer and the concentration was calculated following Jeffrey and Humphrey (1975): Chl-$a$ = (11.85 ($E_{664}$ − $E_{750}$) − 1.54 ($E_{647}$ − $E_{750}$) − 0.08 ($E_{630}$ − $E_{750}$) v)/ V, where $E_{750}$, $E_{664}$, $E_{647}$ and $E_{630}$ are the absorbances at 750, 664, 647 and 630 nm respectively; v is the volume of acetone used in the extraction (ml); and V is the volume of filtered seawater (ml).

The total particulate matter (TPM, mg l$^{-1}$), organic (POM, mg l$^{-1}$) and inorganic (PIM, mg l$^{-1}$) classes of the seston were measured simultaneously with this study in a parallel survey (see Irisarri et al., 2013). Seston quality was expressed as the relative organic content by weight ($Q_1$ = POM/TPM).

## 2.3 Physiological measurements

On each sampling date, mussels between 50 and 60 mm shell length were randomly sampled from rafts P-46 and P-14, cleaned of epibionts and placed in chambers with flowing seawater. Seawater from 3 m depth was supplied by a peristaltic pump to a header tank and filtered through a 50 µm nylon mesh before being distributed at a constant flow rate to the experimental chambers (Filgueira et al., 2006). Physiological measurements were carried out during the morning, coinciding with the period when sea bream were manually fed and presumably, when maximum fish feed-derived POM was exiting the cages. The clearance rate (CR, l h$^{-1}$; n = 280) of mussels was estimated using a flow-through chamber method, to measure the reduction in suspended particles concentration (mm$^3$ l$^{-1}$) as the difference between the inflow and outflow of the chambers (Filgueira et al., 2006). Mussels were placed in cylindrical chambers of 300 ml and left feeding for 1 h before conducting the sampling. Mussels were placed with the inhalant aperture towards the water inflow and the exhalant aperture towards the water outflow. Two experimental chambers were left empty to control for any sedimentation within the chambers.

The CR was calculated following Hildreth and Crisp (1976): CR= f x [(Ci − Co) / Ci], where CR is the clearance rate (l h$^{-1}$), f is the flow rate across the experimental chamber (l h$^{-1}$) and Ci and Co are inflow and outflow particle concentrations (mm$^3$ l$^{-1}$). Particle concentrations within the

2.7 to 40 µm size range were determined with a Coulter Multisizer II fitted with a 100 µm aperture.

The organic ingestion rate (OIR, mg h$^{-1}$; n = 280) was estimated as the product of CR and the particulate organic matter of the seston (POM, mg l$^{-1}$) (OIR = CR x POM). Absorption rate (AR, mg h$^{-1}$; n = 280) was calculated by multiplying the absorption efficiency (AE) by OIR (AR = AE x OIR). The absorption efficiency (AE, %) was calculated according to Conover's ratio (Conover, 1966) after determining the organic and inorganic content of the natural seston and the mussels' feces. The AE data measured simultaneously with this study were reported by Irisarri et al. (2013) and these data are therefore not repeated herein.

Oxygen consumption rate (VO$_2$, ml h$^{-1}$; n = 140) was determined by incubating the mussels in sealed chambers filled with seawater previously filtered through a 30 µm mesh. Chambers were immersed in a seawater bath to maintain temperature conditions similar to the natural environment. One chamber without a mussel was used as a control. The decline in O$_2$ concentration was recorded with YSI$^®$58 oxygen meters connected to YSI$^®$5730 probes until O$_2$ concentration dropped below 30% relative to the control chamber (to avoid hypoxia) (Babarro et al., 2000a). Oxygen consumption was determined as the difference in oxygen concentration between the control and experimental chamber following the equation proposed by Widdows (1985): VO$_2$= 60 [Ct$_o$–Ct$_1$] [V/ t], where Ct$_0$ and Ct$_1$ are the concentrations of oxygen in the water (mg l$^{-1}$) at the start and end of the incubation, respectively, V is the respirometer's volume and t represents the duration (min) of the incubation. Transformations into ml O$_2$ h$^{-1}$ were performed using the equivalents proposed by Widdows (1985): 1 ml O$_2$ = 1 mg O$_2$/ 1.428.

Ammonia excretion rate (VNH$_4$–N, µg h$^{-1}$; n = 140) was determined after placing the mussels in open chambers containing 250 ml of seawater previously filtered through 0.2 µm Millipore membranes. Chambers were kept in a water bath to maintain natural temperature conditions. An empty chamber was used as a control. Water samples were extracted from each beaker after a

150 min incubation period and frozen at –20º C until ammonia analysis, following the phenol–hypochlorite method of Solorzano (1969). Ammonia excretion was calculated as the difference in ammonia concentration between the experimental and control chamber: $VNH_4–N = [(test– control) (14/(1000/V)) (1/t)]$, where V is the volume of the experimental chamber (250 ml) and t is the incubation time (150 min). The ratio of oxygen consumed to nitrogen excreted (O:N), was calculated according to Widdows (1985) as atomic equivalents: $O:N = [O_2 (mL \ h^{-1}) \ x \ 1.428/16] / [NH_4–N \ (mg \ h^{-1}) /14]$.

The CR was standardized for an individual of 60 mm and the $VO_2$ and $VNH_4–N$ were standardized for an individual of 1g soft tissue dry weight: $Y_s = Y_e \ x \ (X_s/X_e)^b$, were $Y_s$ is the standardized (to weigh or to length) physiological rate; $Y_e$ is the experimental physiological rate; $X_s$ is the standard length or body weight and $X_e$ is the length or weight of the experimental animal. Allometric exponents were: b = 0.75 for oxygen consumption and ammonia excretion rate (Bayne and Newell, 1983) and b = 1.85 for clearance rate (Filgueira et al., 2008).

The Scope for Growth (SFG, $J \ h^{-1}$; n=280) was computed following the energy balance equation proposed by Winberg (1960) and Ivlev (1966): SFG = I – F – M = A – M, where I is the ingested energy; F is the energy loss in the feces, M summarizes the respiration and ammonia expenditure; A is the assimilated ration, computed as the product of the I and AE (Labarta et al., 1997). Transformation of physiological rates into energetic units ($J \ h^{-1}$) was performed using the following equivalents; 1 mg POM = 23.5 J, 1 ml $O_2$ = 20.36 J and 1µg $NH_4–N$ = 0.0249 J (Widdows, 1985). Net growth efficiency ($K_2$) was calculated as SFG/AR.

## 2.4. Data analysis

Spatial (raft P-46 *vs* P-14) and temporal (seasonal) environmental and physiological differences were tested with two–way analysis of variance (ANOVA) followed by Tukey's HSD test for multiple pairwise comparisons. ANOVA's assumptions of normality and homogeneity of

variance were checked with Shapiro-Wilk and Levene test, respectively. Non–parametric ANOVA (ANOVA by ranks) was performed when data did not fit normality and homogeneity of variance. Pearson correlation coefficients were calculated to detect significant relationships between the physiological rates and the measured dietary variables. Only the environmental parameters that were significantly correlated with the physiological rates in the Pearson's correlation matrix were included in the regression models. Backwise multiple linear regression analysis was performed to model the environmental parameters predicting the highest variability of the physiological rates. When two or more variables were significant for the model, environmental parameters that did not predict any further variance (i.e. improved the model) were rejected in each step to obtain the most parsimonic and best-fit model. Data analyses were executed in Statistica 7.0 (StatSoft, Inc.).

## 3. Results

### 3.1. Environmental measurements

The seasonal and spatial variation of the environmental measurements is shown in Table 2 and Fig. 2. The average chlorophyll concentration in the Lorbé raft polygon was $0.65 \pm 0.24$ µg l$^{-1}$, with $0.59 \pm 0.26$ µg l$^{-1}$ at the reference raft P-46 and $0.68 \pm 0.20$ µg l$^{-1}$ at raft P-14. Maximum levels were reached in spring ($0.93 \pm 0.23$ µg l$^{-1}$), followed by a drop in the summer ($0.44 \pm 0.12$ µg l$^{-1}$). Phytoplankton size-classes also varied seasonally and presented inter-annual differences. Nanophytoplankton was the most abundant class, comprising 70% of the total chlorophyll at the rafts during the spring-summer period (i.e. upwelling) and 50% during the autumn-winter (i.e. downwelling) (Fig. 3). Micro- and picophytoplankton accounted for 13% and 15% during upwelling and 23% and 24% during downwelling, respectively (Fig. 3). Average maximum values of nanophytoplankton were registered in spring ($0.62 \pm 0.22$ µg l$^{-1}$) and minimum in autumn 2011 ($0.23 \pm 0.16$ µg l$^{-1}$). Pico- and microphytoplankton peaked in spring ($0.16 \pm 0.08$ µg l$^{-1}$ and $0.14 \pm 0.09$ µg l$^{-1}$) and autumn 2011 ($0.21 \pm 0.17$ µg l$^{-1}$ and $0.20 \pm 0.05$ µg l$^{-1}$) and descended in the summer ($0.04 \pm 0.01$ µg l$^{-1}$ and $0.04 \pm 0.02$ µg l$^{-1}$).
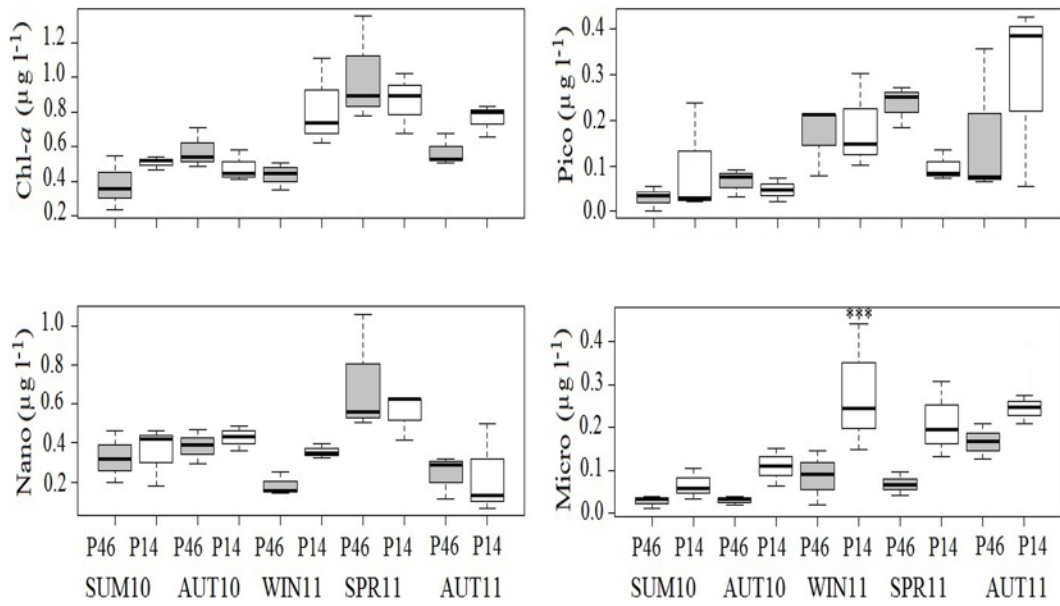
Fig. 2. Box-plots representing the mean seasonal values of chlorophyll-*a* (chl-*a*; µg l$^{-1}$) and phytoplankton size-classes registered at the reference site (gray box; P-46) and the site adjacent to the fish cages (white box; P-14) in Lorbé mussel raft polygon (Galicia, N.W. of Spain). Significant differences are denoted by $P < 0.05^*$, $P < 0.01^{**}$, $P < 0.001^{***}$.
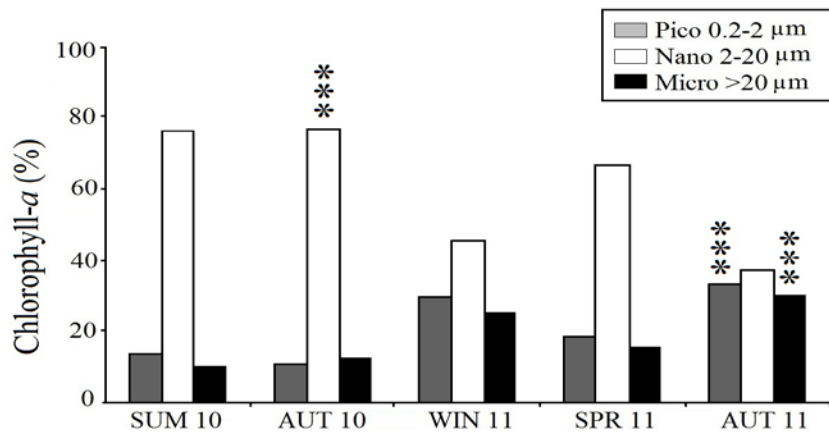


Fig. 3. Seasonal percentage contribution of the phytoplankton size-classes to the total chlorophyll-*a* in Lorbé raft polygon (Galicia, NW Spain). Significant differences are denoted by $P < 0.001^{***}$.

The two–way ANOVA followed by Tukey's test revealed significant temporal differences in the chl-*a* and all phytoplankton classes, with higher levels in spring than in the rest of the seasons (Tukey HSD, *P*<0.001; Table 3; Fig. 2). The spatial differences detected for the microphytoplankton depended on the season, and levels in the raft close to the fish cages (P-14) were higher than at the reference site during winter (Tukey HSD, *P* < 0.001; Fig. 2).

| Effect | df | SS | MS | F-value | P-value |
|---|---|---|---|---|---|
| Chlorophyll-*a* | | | | | |
| Site | 1 | 0.06 | 0.06 | 2.5 | 0.12 ns |
| Season | 4 | 0.83 | 0.21 | 8.03 | < 0.001 *** |
| Site x Season | 4 | 0.29 | 0.07 | 2.76 | 0.06 ns |
| Error | 20 | 0.52 | 0.03 | | |
| Picophytoplankton | | | | | |
| Site | 1 | 0 | 0 | 0.06 | 0.80 ns |
| Season | 4 | 0.14 | 0.03 | 3.22 | < 0.001 *** |
| Site x Season | 4 | 0.06 | 0.01 | 1.37 | 0.28 ns |
| Error | 20 | 0.21 | 0.01 | | |
| Nanophytoplankton | | | | | |
| Site | 1 | 0 | 0 | 0.09 | 0.77 ns |
| Season | 4 | 0.58 | 0.15 | 6.29 | < 0.001 *** |
| Site x Season | 4 | 0.08 | 0.02 | 0.88 | 0.49 ns |
| Error | 20 | 0.46 | 0.02 | | |
| Microphytoplankton | | | | | |
| Site | 1 | 0.08 | 0.08 | 19.03 | < 0.001 *** |
| Season | 4 | 0.11 | 0.03 | 6.42 | < 0.001 *** |
| Site x Season | 4 | 0.02 | 0.01 | 1.38 | 0.27 ns |
| Error | 20 | 0.09 | 0 | | |

Table 3. Results of the two-way ANOVA testing the influence of site, season and the interaction term (site x season) on chlorophyll-*a* and phytoplankton size-classes sampled in Lorbé raft polygon. Significant differences are denoted by *P<0.05, ** P<0.01, *** P<0.001 and ns (not significant).

### 3.2. Physiological measurements

### 3.2.1. Clearance rate (CR), organic ingestion rate (OIR) and absorption rate (AR)

Average CR, OIR and AR registered for *M. galloprovincialis* for both sites and seasons were $2.91 \pm 1.17$ l h$^{-1}$, $1.14 \pm 0.50$ mg h$^{-1}$ and $0.85 \pm 0.42$ mg h$^{-1}$, respectively (Table 2, Fig. 4). ANOVA showed a significant effect of the site on the OIR ($P < 0.05$; Table 4) and a significant effect of the season and the interaction term (site x season) on the CR, OIR and AR (Table 4). Mussels at the raft close to the fish cages had a significantly higher mean annual OIR ($1.24 \pm 0.54$ mg h$^{-1}$) with respect to the reference raft ($1.04 \pm 0.44$ mg h$^{-1}$) (Tukey HSD, $P < 0.001$; Fig. 4). The CR was significantly lower in winter ($2.38 \pm 1.17$ l h$^{-1}$) compared to the other seasons ($3.05 \pm 1.14$ l h$^{-1}$) and was significantly higher at the raft close to the fish cages ($2.49 \pm 0.90$ l h$^{-1}$) compared with the reference site ($2.27 \pm 1.40$ l h$^{-1}$) during winter. The OIR and AR were higher in spring ($1.26 \pm 0.43$ and $1.21 \pm 0.41$ mg h$^{-1}$) and autumn 2011 ($1.29 \pm 0.58$ and $0.95 \pm 0.34$ mg h$^{-1}$) relative to the other seasons (Tukey HSD, $P < 0.001$). The OIR was significantly higher at P-14 than at the reference during winter and autumn 2011 ($P < 0.001$: Fig. 4). However, mussels at raft P-14 had lower CR, OIR and AR in autumn 2010 than P-46 ($P < 0.001$; Fig. 4).

Clearance rate was negatively correlated with seston TPM and PIM, whereas a positive correlation was computed for chl-*a*, the nano-, pico- and microphytoplankton classes and seston quality $Q_1$ (Table 5). The OIR was positively correlated with the chl-*a*, the nano- and microplankton classes, TPM and $Q_1$. The AR was negatively correlated with TPM and PIM, whereas a positive correlation was established with chl-*a* and $Q_1$ (Table 5). The total chl-*a* concentration explained the highest variation in CR (74-86 %; see linear model in Table 6 and Fig. 5), although the nano-, micro- and picoplankton size classes also explained 71-73%, 61-70% and 51-62% of the variation, respectively. Similarly, chl-*a* explained the highest variation in OIR (77-87 %, Table 6; Fig. 5), but the nano- and microplankton classes explained between 63-75% and 62-78%, respectively. Lastly, the highest variability of AR was attributed to the chl-*a* and $Q_1$ (86 %; Table 6; Fig. 5).

| Sampling site | Season | Chl-$a$ $\mu g\ l^{-1}$ | Pico $\mu g\ l^{-1}$ | Nano $\mu g\ l^{-1}$ | Micro $\mu g\ l^{-1}$ | CR $l\ h^{-1}$ | OIR $mg\ h^{-1}$ | AR $mg\ h^{-1}$ | $VO_2$ $ml\ O_2\ h^{-1}$ | $VNH_4$-N $\mu g\ NH_4$-N $h^{-1}$ | O:N ratio | SFG $J\ h^{-1}$ | $K_2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P-46 | Summer 2010 | $0.38 \pm 0.16$ | $0.04 \pm 0.02$ | $0.32 \pm 0.13$ | $0.02 \pm 0.01$ | $3.49 \pm 0.67$ | $1.23 \pm 0.40$ | $0.95 \pm 0.13$ | $2.25 \pm 0.38$ | $25.66 \pm 1.51$ | 111.09 | $-24.11 \pm 7.29$ | $-1.08 \pm 0.94$ |
| P-14 | | $0.51 \pm 0.03$ | $0.09 \pm 0.12$ | $0.35 \pm 0.15$ | $0.07 \pm 0.03$ | $2.33 \pm 0.31$ | $0.94 \pm 0.38$ | $0.73 \pm 0.29$ | $2.99 \pm 1.09$ | $26.35 \pm 4.54$ | 143.37 | $-45.04 \pm 7.03$ | $-2.71 \pm 2.16$ |
| P-46 | Autumn 2010 | $0.58 \pm 0.11$ | $0.05 \pm 0.02$ | $0.42 \pm 0.06$ | $0.11 \pm 0.04$ | $3.43 \pm 0.72$ | $1.14 \pm 0.41$ | $1.01 \pm 0.32$ | $0.48 \pm 0.12$ | $13.96 \pm 2.49$ | 43.7 | $11.66 \pm 7.65$ | $0.54 \pm 0.26$ |
| P-14 | | $0.48 \pm 0.08$ | $0.07 \pm 0.03$ | $0.38 \pm 0.08$ | $0.03 \pm 0.01$ | $2.33 \pm 0.64$ | $0.90 \pm 0.22$ | $0.55 \pm 0.22$ | $0.15 \pm 0.048$ | $6.91 \pm 1.03$ | 28.56 | $10.22 \pm 5.39$ | $0.75 \pm 0.14$ |
| P-46 | Winter 2011 | $0.43 \pm 0.08$ | $0.16 \pm 0.07$ | $0.18 \pm 0.06$ | $0.08 \pm 0.06$ | $2.39 \pm 0.57$ | $0.73 \pm 0.17$ | $0.38 \pm 0.23$ | $0.51 \pm 0.12$ | $14.06 \pm 3.24$ | 47.49 | $-1.79 \pm 5.57$ | $-0.14 \pm 0.09$ |
| P-14 | | $0.82 \pm 0.25$ | $0.18 \pm 0.10$ | $0.35 \pm 0.03$ | $0.28 \pm 0.16$ | $2.51 \pm 0.38$ | $1.27 \pm 0.19$ | $0.05 \pm 0.18$ | $0.30 \pm 0.07$ | $5.03 \pm 1.05$ | 80.38 | $4.09 \pm 3.75$ | $0.39 \pm 0.02$ |
| P-46 | Spring 2011 | $1.00 \pm 0.30$ | $0.23 \pm 0.04$ | $0.70 \pm 0.31$ | $0.06 \pm 0.02$ | $3.01 \pm 0.92$ | $1.11 \pm 0.34$ | $1.10 \pm 0.38$ | $0.61 \pm 0.14$ | $13.68 \pm 3.45$ | 60.97 | $13.08 \pm 8.97$ | $0.50 \pm 0.10$ |
| P-14 | | $0.86 \pm 0.17$ | $0.10 \pm 0.03$ | $0.55 \pm 0.12$ | $0.20 \pm 0.08$ | $3.02 \pm 0.69$ | $1.27 \pm 0.29$ | $1.33 \pm 0.41$ | $0.91 \pm 0.24$ | $13.38 \pm 3.76$ | 91.69 | $12.30 \pm 9.86$ | $0.39 \pm 0.10$ |
| P-46 | Autumn 2011 | $0.59 \pm 0.08$ | $0.17 \pm 0.00$ | $0.24 \pm 0.11$ | $0.16 \pm 0.04$ | $2.60 \pm 0.27$ | $0.95 \pm 0.10$ | $0.84 \pm 0.28$ | $0.24 \pm 0.69$ | $9.11 \pm 2.47$ | 35.24 | $14.78 \pm 6.68$ | $0.70 \pm 0.14$ |
| P-14 | | $0.76 \pm 0.05$ | $0.29 \pm 0.02$ | $0.23 \pm 0.05$ | $0.24 \pm 0.03$ | $3.23 \pm 0.57$ | $1.64 \pm 0.29$ | $1.06 \pm 0.36$ | $0.32 \pm 0.08$ | $2.19 \pm 0.68$ | 196.01 | $18.43 \pm 8.64$ | $0.68 \pm 0.17$ |

Table 2. Means $\pm$ SD of environmental and physiological parameters registered in the reference raft P-46 (further from the net-pens) and P-14 (beside the fish farm) in Lorbé raft polygon (Galicia, N.W. Spain). Environmental parameters included chlorophyll-$a$ (chl-$a$, $\mu g\ l^{-1}$) and the phytoplankton size-classes: picophytoplankton (pico, $\mu g\ l^{-1}$; 0.2-2 $\mu m$), nanophytoplankton (nano, $\mu g\ l^{-1}$; 2-20 $\mu m$), microphytoplankton (micro, $\mu g\ l^{-1}$; > 20 $\mu m$). Physiological rates included the Clearance rate (CR, $l\ h^{-1}$); Organic Ingestion Rate (OIR, $mg\ h^{-1}$); Absorption rate (AR, $mg\ h^{-1}$); ($VO_2$, $ml\ O_2\ h^{-1}$); Ammonia excretion rate ($VNH_4$-N, $\mu g\ NH_4$-N $h^{-1}$); O:N ratio; Scope for Growth (SFG, $J\ h^{-1}$) and net growth efficiency ($K_2$).

### 3.2.2. Respiration rate ($VO_2$), ammonia excretion rate ($VNH_4$–N) and O:N ratio

Mean $VO_2$, $VNH_4$–N and O:N index registered for *M. galloprovincialis* at both sites and all seasons were $0.85 \pm 0.42$ ml h$^{-1}$, $12.70 \pm 4.9$ µg h$^{-1}$ and 86.93 (Table 2, Fig. 4). A significant effect of the site, season and the interaction term (site x season) was observed for $VO_2$, $VNH_4$–N and O:N ratio (ANOVA, $P < 0.05$; Table 4). Mussels from the reference raft had significantly higher mean annual $VNH_4$–N ($14.69 \pm 5.64$ µg h$^{-1}$) than mussels close to the fish cages ($10.60 \pm 9.30$ µg h$^{-1}$). However, mussels from the latter site had significantly higher $VO_2$ and O:N ratio ($0.96 \pm 1.10$ ml h$^{-1}$, 116.79) than mussels from the reference ($0.74 \pm 0.67$ ml h$^{-1}$, 57.51) (Tukey HSD, $P < 0.001$; Fig. 4). $VO_2$, $VNH_4$–N and O:N ratio showed significantly higher values in the summer ($2.67 \pm 0.71$ ml h$^{-1}$, $26.06 \pm 3.56$ µg h$^{-1}$ and 127.23) than the rest of the seasons (Tukey HSD, $P < 0.001$). Mussels distant from the fish farm displayed significantly higher $VO_2$ and $VNH_4$–N than P-14 in winter and autumn 2010 and 2011, excepting for no differences detected for $VO_2$ in autumn 2011. However, mussels close to the net-cages had higher oxygen consumption than the reference in spring. The O:N ratio was higher in the raft close to the net-pens than P-46 in winter, spring and autumn 2011 (Tukey HSD, $P < 0.001$; Fig. 4).

The $VO_2$ and $VNH_4$–N were negatively correlated with the chlorophyll and the phytoplankton size-classes (Table 5). The nanophytoplankton size-fraction explained the highest variation in $VO_2$ (43 %; Table 6, Fig. 5), although the chl-*a* also explained between 33 and 37% of the variability. Similarly, nanoplankton explained the highest variation in $VNH_4$–N (67-70%; Table 6; Fig. 5), whereas the chl-*a* explained between 45 and 69% of the variation.

| | Site | | | | Season | | | | Site x season | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SS | MS | F | P | SS | MS | F | P | SS | MS | F | P |
| Clearance rate | 0.43 | 0.43 | 0.45 | 0.49 ns | 21.86 | 5.46 | 5.79 | < 0.001 *** | 37.16 | 9.29 | 9.85 | < 0.001 *** |
| Organic ingestion rate | 64604 | 64604 | 17.07 | < 0.01 ** | 149321 | 37330 | 9.86 | < 0.001 *** | 358531 | 89633 | 23.69 | < 0.001 *** |
| Absorption rate | 8930 | 8930 | 2.53 | 0.11 ns | 742852 | 185713 | 52.62 | < 0.001 *** | 205138 | 51285 | 14.53 | < 0.001 *** |
| Respiration rate | 38.72 | 3872 | 6.08 | < 0.05 * | 507667 | 126917 | 199.5 | < 0.001 *** | 112384 | 28096 | 44.16 | < 0.001 *** |
| Ammonia excretion rate | 57113 | 14278 | 239.37 | < 0.001 *** | 20492 | 20492 | 343.52 | < 0.001 *** | 5330 | 1332 | 22.34 | < 0.001 *** |
| O:N ratio | 29784.29 | 29784.28 | 77.9 | < 0.001 *** | 72501.76 | 18125.44 | 47.4 | < 0.001 *** | 48740.44 | 12185.11 | 31.87 | < 0.001 *** |
| Scope for growth | 1004 | 1004 | 0.5 | 0.48 ns | 999781 | 249945 | 124.59 | < 0.001 *** | 22971 | 5743 | 2.86 | 0.06 ns |
| Net growth efficiency | 10003 | 10003 | 6.29 | < 0.001 *** | 1370168 | 342542 | 215.63 | < 0.001 *** | 86863 | 21716 | 13.67 | < 0.001 *** |

Table 4. Results of the two-way ANOVA testing the influence of site, season and the interaction term (site x season) on the physiological rates measured in Lorbé. Significant differences are denoted by $*P < 0.05$, $** P < 0.01$, $*** P < 0.001$ and ns (not significant).

|  |  | Chl-$a$ | Pico | Nano | Micro | TPM | POM | PIM | $Q_1$ |
|---|---|---|---|---|---|---|---|---|---|
| CR | P-46 | 0.260** | 0.186* | 0.195* | 0.188* | -0.218** | 0.085 | -0.208* | 0.160* |
|  | P-14 | 0.232** | 0.181* | 0.190* | 0.175* | -0.152* | 0.149 | -0.119* | 0.157* |
| OIR | P-46 | 0.419*** | -0.154 | 0.280*** | 0.177* | 0.340*** | 0.224** | -0.332*** | 0.243** |
|  | P-14 | 0.464*** | 0.462*** | 0.243** | 0.475*** | 0.165** | 0.480*** | 0.127 | -0.050 |
| AR | P-46 | 0.380*** | -0.023 | 0.489*** | -0.086 | -0.593*** | 0.516*** | -0.589*** | 0.543*** |
|  | P-14 | 0.393*** | 0.198* | 0.070 | 0.232** | -0.329*** | 0.151 | -0.371*** | 0.479*** |
| VO$_2$ | P-46 | -0.318** | -0.350** | -0.390*** | -0.722*** | -0.074 | 0.052 | -0.073 | -0.054 |
|  | P-14 | -0.520*** | -0.412*** | -0.425*** | -0.561*** | -0.481*** | -0.524*** | -0.465*** | 0.455*** |
| VNH$_4$-N | P-46 | -0.302*** | -0.270* | -0.455*** | -0.722*** | 0.049 | -0.094 | -0.052 | -0.172 |
|  | P-14 | -0.510*** | -0.616*** | -0.425*** | -0.629*** | -0.569*** | -0.666*** | -0.546*** | 0.592*** |
| SFG | P-46 | 0.561*** | 0.333*** | 0.297*** | 0.646*** | -0.293*** | 0.274*** | -0.292*** | 0.403*** |
|  | P-14 | 0.486*** | 0.395*** | 0.143* | 0.439*** | 0.206** | 0.383*** | 0.181* | -0.178* |
| K$_2$ | P-46 | 0.500*** | 0.189* | 0.288*** | 0.596*** | -0.415*** | 0.383*** | -0.414*** | 0.487*** |
|  | P-14 | 0.374*** | 0.277*** | -0.09 | 0.348*** | 0.243** | 0.295*** | 0.230* | -0.233** |

Table 5. Pearson's correlation coefficients for the relationship between the physiological rates and the environmental variables measured at the reference raft (P-46) and the raft beside the net-pens (P-14). Significant levels were denoted as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

| Rate | Site | Best-fit linear model | $R^2$ | P |
|---|---|---|---|---|
| CR | P-46 | CR = 4.27 chl-$a$ | 0.748 | <0.001*** |
|  | P-14 | CR = 3.96 chl-$a$ | 0.861 | <0.001*** |
| OIR | P-46 | OIR=1.51 chl-$a$ | 0.772 | <0.001*** |
|  | P-14 | OIR=1.77 chl-$a$ | 0.873 | <0.001*** |
| AR | P-46 | AR=0.11 chl-$a$ + 1.08 $Q_1$ | 0.867 | <0.001*** |
|  | P-14 | AR=0.67 chl-$a$ + 0.71 $Q_1$ | 0.861 | <0.001*** |
| VO$_2$ | P-46 | VO$_2$=1.46 nano | 0.438 | <0.001*** |
|  | P-14 | VO$_2$=2.61 nano | 0.438 | <0.001*** |
| VNH$_4$-N | P-46 | NH$_4$-N=30.92 nano | 0.700 | <0.001*** |
|  | P-14 | NH$_4$-N= 28.58 nano | 0.676 | <0.001*** |
| SFG | P-46 | SFG= -20.46 + 38.71 chl-$a$ + 0.593 $Q_1$ | 0.305 | <0.001*** |
|  | P-14 | SFG= -36.81+ 68.46 chl-$a$ - 15.38 $Q_1$ | 0.255 | <0.001*** |

Table 6. Linear regressions established between the physiological rates of *M. galloprovincialis* and the characteristics of the seston (particulate organic matter: POM; seston quality: $Q_1$) and the chlorophyll-$a$ and phytoplankton size-classes.

### 3.2.3 Scope for Growth (SFG) and net growth efficiency ($K_2$)

The mean annual SFG and $K_2$ of *M. galloprovincialis* measured at both rafts were $2.94 \pm 19.71$ J h$^{-1}$ and $-0.07 \pm 2.45$ (Table 2, Fig. 4). However, the mean annual SFG and $K_2$ showed higher values ($10.75 \pm 9.47$ J h$^{-1}$ and $0.38 \pm 0.55$) when the negative values registered during the summer survey were omitted. The site, season and interaction term (site x season) exerted a significant effect on the $K_2$, while only the season had a significant effect on the SFG (ANOVA, $P < 0.05$; Table 4). The mean annual $K_2$ of mussels held further from the fish cages ($-0.03 \pm 0.97$) was higher with respect to the raft close to the cages ($-0.12 \pm 1.68$) (Tukey HSD, $P < 0.001$; Fig. 4). The SFG and $K_2$ were significantly lower in the summer ($-34.57 \pm 12.55$ J h$^{-1}$ and $-2.27 \pm 1.90$) than in the other seasons ($10.75 \pm 9.47$ J h$^{-1}$ and $0.38 \pm 1.00$) (Tukey HSD, $P < 0.001$). The $K_2$ of the mussels in the proximity to the fish cages was higher than the reference during winter and autumn 2010 (Tukey HSD, $P < 0.001$; Fig. 4).

|  | B | SE of B | *t* | P |
|---|---|---|---|---|
| *SFG P-46* | | | | |
| Intercept | -20.46 | 3.64 | -5.61 | <0.001** |
| Chl-*a* | 38.71 | 6.92 | 5.59 | <0.001*** |
| Q$_1$ | 0.59 | 6.57 | 0.09 | <0.05* |
| | | | | |
| R$^2$ = 0.315; Adjusted R$^2$ = 0.305; n = 138; F(2,137) = 31.75; *P* < 0.001 | | | | |
| | | | | |
| *SFG P-14* | | | | |
| Intercept | -36.81 | 8.18 | -4.49 | <0.001** |
| Chl-*a* | 68.46 | 10.23 | 6.69 | <0.001** |
| Q$_1$ | -15.38 | 6.50 | -2.36 | <0.05* |
| | | | | |
| R$^2$ = 0.265; Adjusted R$^2$ = 0.255; n=141; F(2,140) = 25.47; *P* < 0.001 | | | | |

Table 7. Summary of the multiple linear regressions models obtained for the Scope for Growth of mussels at Lorbé raft polygon and the variance explained by the chlorophyll-*a* and the seston quality $Q_1$.

The SFG was positively correlated with the chl-*a*, the phytoplankton size-classes and the food quality $Q_1$ (see Pearson's correlations, Table 5). Multiple regression analyses identified the chl-*a* and $Q_1$ as the environmental variables explaining the highest variation of the SFG at P-46 ($R^2$ =0.30) and P-14 ($R^2$ =0.25) (Table 6 and 7; Fig. 5).
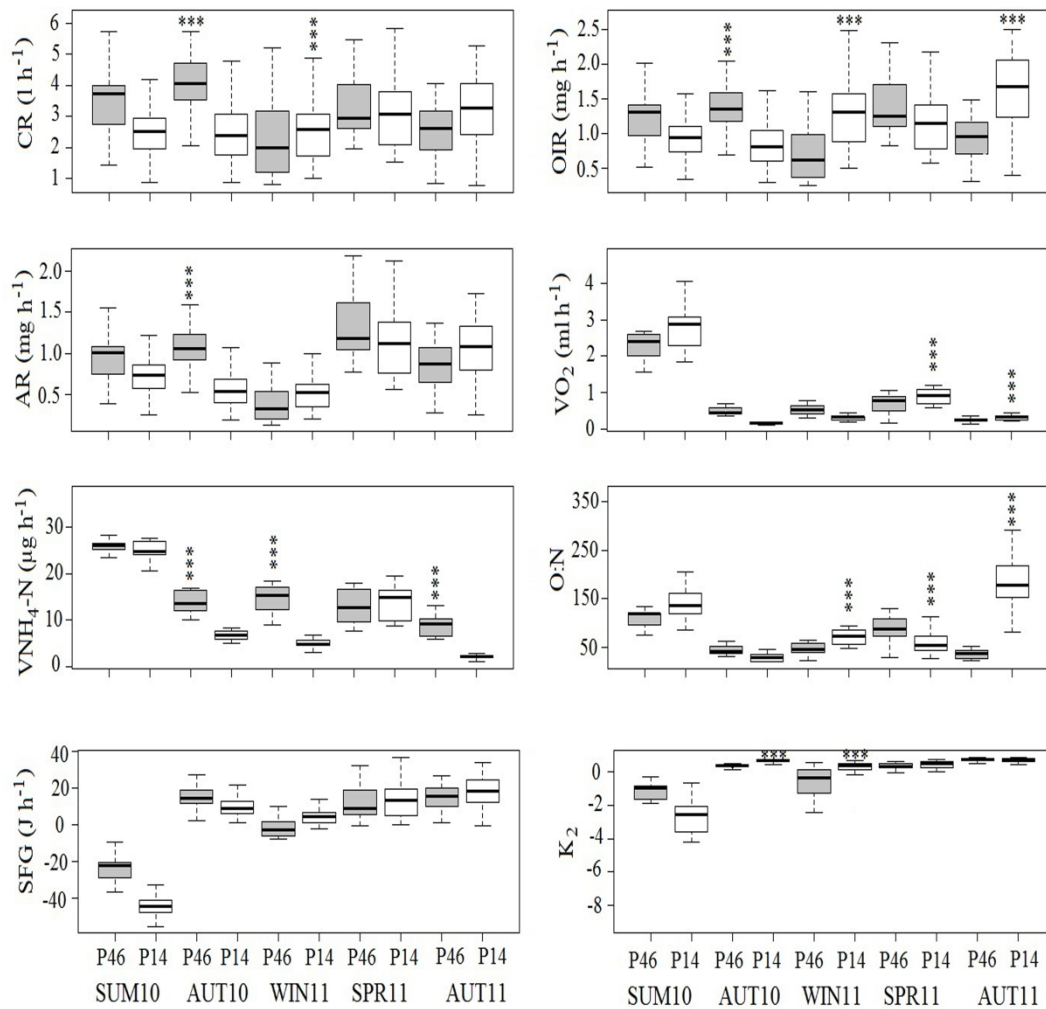


Fig. 4. Box-plots representing the mean seasonal values of the physiological rates of *Mytilus galloprovincialis* at the reference site (gray box; P-46) and the site adjacent to the fish cages (white box; P-14). Significant differences are denoted by $P < 0.05$*, $P < 0.01$**, $P < 0.001$***.

# 4. Discussion

## 4.1. Environmental parameters

### 4.1.1 Seasonal variations

The seasonal pattern of chlorophyll-*a* in this study reflected the upwelling and downwelling events of the Galician Rías (Figueiras et al., 2002; Peteiro et al., 2011), where average chlorophyll is $< 5$ µg l$^{-1}$ (Figueiras et al., 2002). The sampling schedule of this study illustrated the occurrence of a chl-*a* peak in spring, and two troughs in summer and winter. The main peak registered in May was characteristic of the spring phytoplankton bloom, observed in this region during the same timing by Peteiro et al. (2006). The autumn corresponds with the transition period between the summer upwelling and the winter mixing, when the reversal circulation of the Rías favors phytoplankton accumulation (Figueiras et al., 2002). The significantly lower chl-*a* concentration in autumn 2010 in comparison with 2011 highlighted the inter-annual variability of the wind patterns and duration of thermal stratification events in the summer (Figueiras et al., 2002; Villegas-Ríos et al., 2011). Minimum chl-*a* levels found in the summer were likely associated with stratification events that occur when the thermocline layer prevents nutrients to fertilize the surface and chl-*a* becomes eventually depleted. Levels of chl-*a* during winter ($> 0.6$ µg l$^{-1}$) were higher than the summer minima ($0.4$ µg l$^{-1}$) suggesting that some nutrients were still available in February as a result of vertical mixing of the surface with deeper layers.

Temporal variations of the phytoplankton size-classes corresponded with the upwelling–downwelling cycle. Nanophytoplankton (2-20 µm) was the most abundant fraction during the whole annual cycle. Concurrently, Tilstone et al. (1999) determined that nanophytoplankton was responsible for the greatest variation in primary production in the Rías, due to higher light utilization efficiency than net phytoplankton. However, the Rías have been traditionally considered as microplankton ($> 20$ µm) dominated ecosystems (Figueiras et al., 2002). Several studies reported a dominance of microphytoplankton during upwelling, and larger abundance of nano- and picoplankton size-classes during downwelling (Tilstone et al., 1999, Cermeño et al.,

2006; Arbones et al., 2008). Arbones et al. (2008) reported that microplankton represented 77-88% of the total chlorophyll during upwelling and 20-79% during downwelling, while nanoplankton were much lower than in our study and varied between 11-18% and 19-74%, respectively. Studies analyzing the abundance of the phytoplankton size-classes in extensive shellfish aquaculture sites are very scarce. Cranford et al. (2008) concluded that picophytoplankton (0.2-2 µm) could become the dominant fraction (50-80% of the total) in poorly flushed, high-density mussel aquaculture sites, where the rate of phytoplankton renewal is lower than the consumption rate by mussels. Picophytoplankton is too small to be captured by mussels, while picophytoplankton predators (ciliates and flagellates) are effectively ingested by mussels (Cranford et al., 2008; 2009a). Similarly, Saffi and Gibbs (2003) observed that micro- (>20 µm) and picophytoplankton size-classes contributed to the largest phytoplankton biomass in Beatrix Bay (New Zealand), as the grazing pressure exerted by the mussel *Perna canaliculus* may have reduced the levels of nanophytoplankton (2-20 µm). The dominance of the nanophytoplankon in the present study reinforced a previous investigation that suggested that mussel farming has not exceeded the production carrying capacity of the Galician Rías at the current raft scale (Duarte et al., 2008). Thus, the phytoplankton supply in Lorbé seemed to be replaced faster by the water flushing and primary production than it was being cleared by the mussels.

### 4.1.2. Spatial variations

This study showed no evidence of chlorophyll-*a* enhancement at the station adjacent to the fish cages in comparison with the reference location in Lorbé raft polygon (Ría Ares-Betanzos, Galicia, NW Spain). Moreover, the results demonstrated no spatial differences for the nano- and picophytoplankton size-classes between the raft stations. The significantly higher levels of microphytoplankton (>20 µm) found at the raft close to the fish farm during winter likely resulted from the lower mussel population (1,440 x $10^3$ mussels) and hence lower grazing pressure exerted by mussels from this raft compared with the reference (4,216 x $10^3$ mussels).

Factors such as high current speed, strong wind action, sediment resuspension events, and/or the feeding action of filtering organisms are considered crucial for the dispersal and dilution of any localized suspended particulate enhancement due to fish farming activity (Troell and Norberg, 1998; Troell et al., 2003, 2011; Cheshuk et al., 2003). Localized chlorophyll enhancement near fish cages also depends on the turnover time of the phytoplankton as well as the stage of the fish production cycle, the stocking density and the volume of dissolved inorganic nutrients (ammonium and phosphorous) released by the net-pens that could be taken up by phytoplankton (Troell and Norberg, 1998; Troell et al., 2003, 2011; Cheshuk et al., 2003). In addition, fish farms located in areas with excess ambient nutrients (i.e. nitrogen) are also less likely to experience a localized enhancement in chlorophyll production due to enrichment by fish inorganic nutrients (Cheshuk et al., 2003). Several studies have reported elevated chlorophyll-*a* levels in the vicinity of fish farms (Jones and Iwama, 1991; Stirling and Okumus, 1995; Dalsgaard and Krause-Jensen, 2006; Pitta et al., 2009; Sarà et al., 2011, 2012). Even if current measurements were not reported in these studies, the authors suggested that the low energetic conditions of the study sites may facilitate the enhancement of chlorophyll in the water surrounding the fish cages (Dalsgaard and Krause-Jensen, 2006; Sarà et al., 2011, 2012).

However, fish farms are generally located in areas with a rapid flushing time that ensures an efficient water renewal. The lack of chlorophyll enhancement beside the fish farm in this study likely owed to the energetic hydrodynamic characteristics of the site. Similarly, no increases of particulate organic matter were observed at 170 m from the fish cages in a simultaneous study (Irisarri et al., 2013). The present study along with the simultaneous survey of Irisarri et al. (2013) measured the availability of feed fines and/or natural seston present in the water column at 3 m depth during the morning fish-feeding period. Given that the fish cages were 6 m depth it might be hypothesized that an increase in feed particles or chlorophyll may occur in shallower or deeper water. However, weekly samples of chlorophyll and seston taken at 1 and 6 m depth at both rafts during a 9 month period showed no significant differences among stations (Irisarri et al., 2014). The results of this study are in agreement with other papers that detected no

evidence of increased chl-*a* near fish cages compared to reference stations (Taylor et al., 1992; Mazzola and Sarà, 2001; La Rosa et al., 2002; Cheshuk et al., 2003; Pitta et al., 1999, 2005; MacDonald et al., 2011; Handå et al., 2012a). Similarly, La Rosa et al. (2002) reported no differences for picophytoplankton density and biomass between a fish farm, a mussel farm and control site in the Tyrrhenian Sea. Concurrently, Troell and Norberg (1998) observed that only when water currents were slow (3-5 cm s$^{-1}$) fish particle concentrations were above 0.1 mg l$^{-1}$. Under high energetic conditions, nutrients released by the fish are diluted in the large volumes of water passing through the cages and any chlorophyll enhancement may be produced away from mussels held adjacent to the fish farm. In this study, the raft beside the fish cages experienced periods of high current speeds, which ranged up to 13 cm s$^{-1}$ during the 2010-2011 sampling (Zuñiga et al., 2014). Moreover, the Ría-Ares Betanzos is a well flushed embayment, with a flushing time of approximately 1 week (Villegas-Ríos et al., 2011). It is likely that the turnover time of the phytoplankton (several days) in Lorbé was longer than the flushing time of the water body. Hence, the action of fast currents coupled with a rapid flushing time may aid to disperse the organic and inorganic nutrients coming from the fish cages and did not favor localized chlorophyll enhancement. Concurrently, Cheshuk et al. (2003) found no significant differences in chlorophyll levels between salmon cages and control sites, as currents up to 24.7 cm s$^{-1}$ rapidly disperse fish-farm nutrients away from the fish farm. Similarly, Handå et al. (2012a) noted that high current speeds at mussel sites near the fish cages (20 and 25 cm s$^{-1}$) transport fish nutrients too quickly to be assimilated by phytoplankton and produce any local chl-*a* enhancement. Furthermore, any enhancement in chlorophyll in this study may also be rapidly grazed by the large mussel stock cultured in the Lorbé raft polygon. Local reduction of chlorophyll in mussel farms mainly depends on hydrodynamic regimes and tidal exchange, as well as natural primary productivity and the clearing action of mussels and the associated epifauna (Cranford et al., 2008).

## 4.2. Physiological rates

### 4.2.1. Seasonal variations

The feeding (CR, OIR) and digestive (AR) rates of *M. galloprovincialis* were comparable with values obtained for this species held directly on rafts (Navarro et al., 1991; Babarro et al., 2000b; Iglesias et al., 1996; Zuñiga et al., 2013) or under laboratory conditions (Labarta et al., 1997; Filgueira et al., 2009, 2010), with length-standardized CR, OIR and AR ranging between 1.31-5.35 l h$^{-1}$, 0.8-2.7 mg h$^{-1}$ and 2.54-3.15 mg h$^{-1}$ in the aforementioned studies. The feeding and digestive rates of *M. galloprovincialis* presented their maximum values during the chlorophyll peak in spring while minimum values were during winter downwelling. Results showed that the CR was negatively affected by increasing TPM and PIM (i.e. lower feeding during periods of reduced seston quality), while a positive correlation was obtained with $Q_1$ and chl-*a*. Similarly, a significant negative correlation (r = –0.54) was established between the CR of *M. edulis* and TPM by Bayne and Widdows (1978), while a positive correlation (r = 0.52) was observed between the CR of the scallop *Placopecten magellanicus* and chl-*a* (MacDonald and Ward, 1994). In this study, CR varied as function of the chl-*a*, which explained 74 to 86% of the variance. This was consistent with models established by Filgueira et al. (2009; 2010), in which the chl-*a* explained 72 to 85% of the variance observed for the CR of *M. galloprovincialis*. The CR of *M. edulis* was also a function of chl-*a* in the study of Strohmeier et al. (2009) and explained 34% of the variance. Variations in CR for *M. galloprovincialis* have also been attributed to the quality of the seston $Q_1$ ($R^2$ = 0.43) (Gardner 2002; Helson and Gardner 2007) and the TPM ($R^2$ = 0.36) (Galimany et al., 2011). Our results agreed with Filgueira et al. *(*2010) that described CR as more strongly influenced by changes in seston quality ($Q_1$, chlorophyll contained in the seston) than quantity (TPM) under the relatively low seston concentrations typically found in the Galician Rías (< 5 mg l$^{-1}$).

Given that seston concentration in the Rías is below the threshold of pseudofeces production, the OIR followed the pattern of the CR. The increases in OIR and AR were also related with the abundance of chl-*a* during the spring and autumn blooms. This was demonstrated by the

positive correlations between the OIR and $Q_1$ and AR with chl-*a*. Significant positive correlations between OIR and TPM, AE and $Q_1$ and AR and $Q_1$ have been also documented for *M. galloprovincialis* and *Perna viridis* (Babarro et al., 2000b; Irisarri et al., 2013; Wong and Cheung, 2001, 2003). In this study, the seasonal variations in OIR and AR were a function of the chl-*a* and $Q_1$. This concurred with studies that attributed 32% of the variance in OIR to the chl-*a* available for *M. galloprovincialis* (Zuñiga et al., 2013). Likewise, the TPM accounted for 51% and 72% of the variance in OIR in the same species (Babarro et al., 2000a; Fernández-Reiriz et al., 2007). Although the highest degree of variation of the feeding rates (CR and OIR) was attributed to the chlorophyll, results further suggested that the mussels' feeding activity was size-specific. The nano- (2-20 μm) and microphytoplankton size-classes (>20 μm) explained a higher proportion of the variability than the picophytoplankton (0.2-2 μm), suggesting that the nano- and microphytoplankton were the size classes being preferentially cleared and ingested by the mussels. A concurrent study on raft-scale phytoplankton depletion in mussel farms in the Lorbé region (Cranford et al., 2014) showed that picophytoplankton were not captured by mussels during passage through rafts, while an average of 40% of phytoplankton between 3 and 50 µm diameter was depleted by the mussels (also see Petersen et al., 2008). Similarly, Fournier et al. (2012) reported that the *in situ* clearance rate of the oyster *Pinctada margaritifera* was higher for nano- and microphytoplankton size classes than for picophytoplankton.

The metabolic rates ($VO_2$, $VNH_4$–N) and O:N resembled previous studies (Navarro et al., 1991; Labarta et al., 1997; Babarro et al., 2000a; Zuñiga et al., 2013) that reported $VO_2$ between 0.36 and 1.4 ml h$^{-1}$, $VHN_4$–N ranging from 0.88 to 21.7 µg h$^{-1}$ and O:N ratio between 39 and 116. The $VO_2$ and $VNH_4$–N presented their maximum values during the summer stratification, while minimum values were during the autumn chlorophyll peak. This was supported by the negative correlation observed between $VO_2$ and $VNH_4$–N with the chlorophyll and the phytoplankton size-classes (i.e. high respiration and excretion under depleted chlorophyll levels). In addition, based on the abnormally high water temperature recorded in July (17.5 ℃ *vs* July average of 14-15 ℃), mussels could have experienced a heat shock that lead metabolic rates to exceed energy acquisition. Concurrently, in Alfacs Bay, suspended *M. galloprovincialis* reduced the clearance,

ingestion and absorption rates during the high temperatures registered in July (Galimany et al., 2011). Fluctuations of $VO_2$ in *M. galloprovincialis* and *M. edulis* have been related with food availability, temperature, salinity and reproductive condition (Babarro et al., 2000a; Sarà and Pusceddu, 2008; Handå et al., 2013). In this study, the $VO_2$ and $VNH_4$–N appeared to vary mainly as functions of the food supply (particularly the nanophytoplankton and chl-*a*). Similarly, the chl-*a* also explained 69% of the variability of $VO_2$ in the study of Babarro et al. (2000a), who also observed that the chl-*a* explained part of the variability of $VNH_4$–N in the same study. On the other hand, other studies found that variations in $VO_2$ and $VNH_4$–N in bivalves *Mytilus chilensis* and *Mulinia edulis* were explained by the TPM and POM contained in the seston (Velasco and Navarro, 2003).
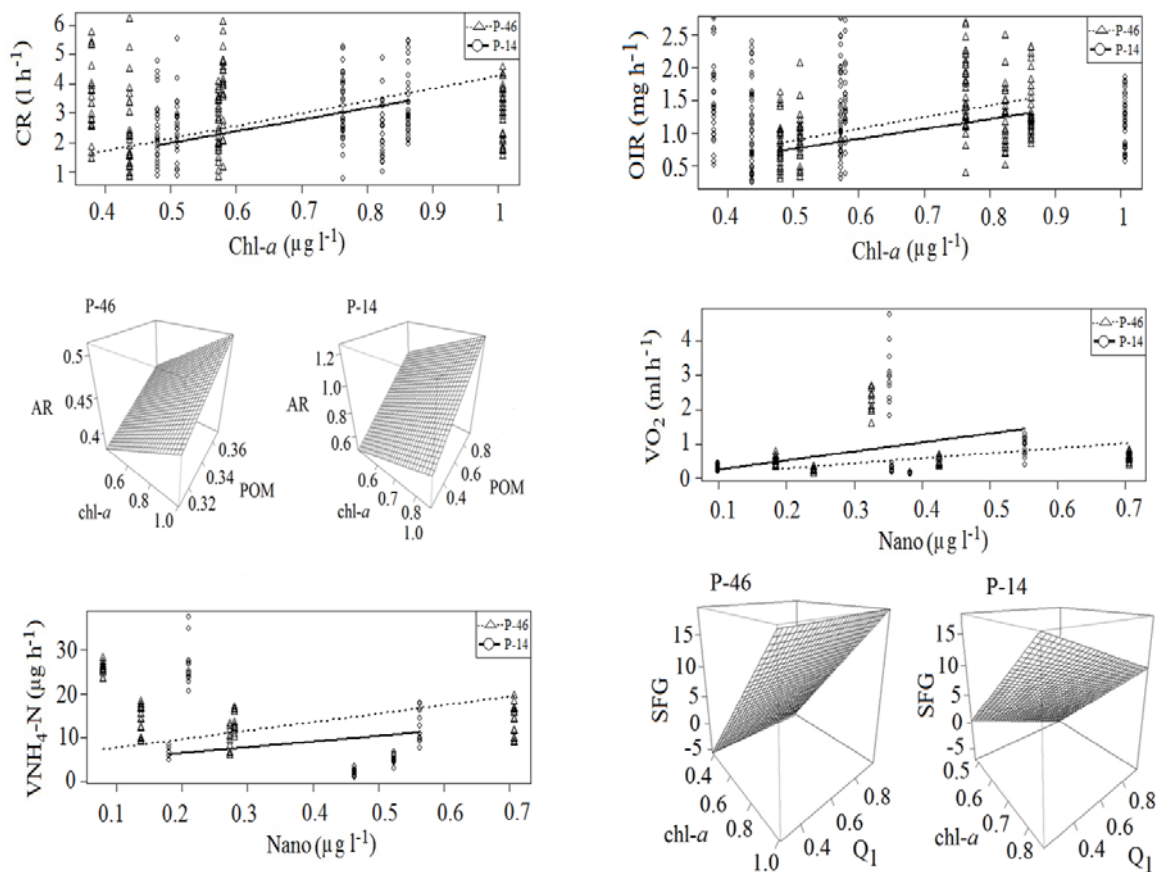


Fig. 5. Relations between the physiological rates of *M. galloprovincialis* with the environmental variables measured simultaneously in the field.

The seasonal variations in the O:N ratio integrate the fluctuations in respiration and ammonium excretion and provide information on substrate catabolism. The significantly higher O:N ratio during the summer was probably caused by the catabolism of energetic reserves (i.e., glycogen and lipids) rather than the thermal stress, since the O:N ratio usually declines during high temperatures (Bayne et al., 1985; Handå et al., 2013). Hence, we suggest that mussels had stored reserves during the spring bloom that resulted in a high O:N ratio during the summer.

The Scope for Growth (SFG) and net growth efficiency ($K_2$) of *M. galloprovincialis* were lower than the range between $-2.78$-37 J h$^{-1}$ and $-0.45$-0.82 reported in other studies (Navarro et al., 1991; Labarta et al., 1997; Albentosa et al., 2012), although these differences were minimal if the significantly lower values obtained during the summer stressful temperatures were excluded. The SFG had positive mean values during the spring and autumn bloom, decreased during the winter mixing and reached negative values during the summer stratification. The net growth efficiency ($K_2$)–the effectiveness with which food is turned into body tissues– showed a similar pattern. The seasonality of the SFG of *M. galloprovincialis* was best explained by fluctuations in food quality ($Q_1$) and chlorophyll. Similarly, a significant positive correlation between the SFG and $Q_1$ was observed for the mussel *P. viridis* (Wong and Cheung, 2003). The positive SFG measured during spring and autumn indicated that there was energy available for growth, and it was correlated with the high nutritional value of the seston (96.3% organic content) during the chlorophyll peaks, resulting in higher energy acquisition (CR, OIR, AR) and lower energy expenditure (VO$_2$, VNH$_4$–N). On the other hand, the low chlorophyll levels and stressful temperatures registered in the summer survey resulted in a negative SFG, as the energy consumed and absorbed by the mussels was lower than the energy required for respiration and excretion. The variability of the SFG explained by the chl-*a* in this study was in accordance with the value reported by Pérez-Camacho et al. (1995) for the individual growth of *M. galloprovincialis* (21-33%) cultured in rafts in a Galician Ría. Similarly, Wong and Cheung (2003) reported that the SFG of *P. viridis* showed a positive relationship with $Q_1$ ($R^2 = 0.42$). Thus, our results support numerous studies that have demonstrated that the SFG of mussels

depends on the food quality and quantity of the seston (Wong and Cheung, 2001, 2003; Velasco and Navarro, 2003; Helson and Gardner, 2007; Sarà and Pusceddu, 2008).

### 4.2.2. Spatial variations

The results demonstrated that mussels grown close to the fish net-pens had similar feeding, digestive and metabolic rates compared to mussels at the reference location. Hence, proximity to the fish cages did not result in higher energy for growth or reproduction for the mussels. The similar concentrations of chl-*a*, seston quantity and quality among both sites resulted in comparable physiological rates, SFG and $K_2$. These results were in agreement with the field experiment of Cheshuk et al. (2003), who reported no differences in growth for mussels kept at reference sites and near salmon cages due to the absence of significant organic matter and chlorophyll enhancements near the fish cages. Similarly, Navarrete-Mier et al. (2010) also obtained no enhancement in the growth of *M. galloprovincialis* cultured in the proximity of an open-water fish farm. In contrast, surveys reporting a significant growth enhancement close to fish cages also measured increases in chlorophyll levels and/or particulate organic matter (Sarà et al., 2009, 2012). Sarà et al. (2012) detected greater maximum length, growth rates and faster maturation of mussels *M. galloprovincialis* cultured near fish cages due in part to 45% greater chlorophyll levels than at a monoculture site.

To our knowledge, the findings in this study represent the first attempt to assess the spatial differences in Scope for Growth of bivalves reared adjacent to fish cages. The clearance rate and absorption efficiency have been considered the main process responsible of energy acquisition in bivalves (Hawkins et al., 1999), and the physiological rates that most influence the determination of SFG (Albentosa et al., 2012). In this study, the CR of *M. galloprovincialis* held beside the fish cages was similar to that of mussels cultured at the reference station, except for higher CR at the raft close to the fish cages during winter and autumn resuspension events (see Irisarri et al., 2013). Short-term reductions in the quality of the seston during this time lead to significantly lower absorption efficiency (Irisarri et al., 2013), which was likely compensated by

enhancing the CR. Previous studies with *M. edulis* reported similar clearance rates (~2-3 $1\,h^{-1}$) for fish feed, fish feces and microalgae diets supplied under laboratory conditions (MacDonald et al., 2011; Handå et al., 2012b). Unlike experiments performed under laboratory conditions, the open-water scenario used in this study makes it difficult to prove that mussels were filtering fish farm effluents, as fish particles and seston become mixed in the marine environment. Nonetheless, even if mussels cleared some of the fish particles, this was not reflected in a higher SFG. If we consider that the majority of fish suspended solids are < 40 µm ESD (MacDonald et al., 2011; Lander et al., 2013) and that mussels have been reported to effectively retain particles of 3-50 µm in the Lorbé region (Cranford et al., 2014), a large fraction of the particulate effluents could be potentially cleared. However, a recent study suggested that approximately only 0.6 to 1.8% of the fish particles would be effectively incorporated into the mussels' biomass (Wang et al., 2012). The comparable physiological rates and SFG found adjacent to the cages and reference location suggest that mussels will only utilize a potential enhancement in chlorophyll and fish particulate wastes when the integrated culture is performed in areas that combine adequate husbandry practices, hydrodynamics and seston concentrations (Troell et al., 2011).

Several studies have demonstrated that the main particulate plume of fish wastes occurs within 50 to 60 m from the fish farm and after this distance wastes are dispersed and diluted to ambient levels (Cheshuk et al., 2003; Lander et al., 2013). In this study, the placement of bivalves 170 m from the fish cages would prevent the mussels from being exposed to relatively high concentrations of fish wastes, although the high density of mussel rafts in the regions would likely place mussels within the transport pathway of much of the fine suspended waste. The position of the rafts and fish cages in this study could not be altered, since this is regulated through specific fish and shellfish management plans for coastal areas. Consequently, the location of the rafts relative to the fish pens was not based on any optimal design for fish waste exploitation by mussels. The rate of dispersion of particulate wastes, as well as the efficiency of particle depletion by mussels depends on the local current speed (Cranford et al., 2013). Cranford et al. (2013) estimated that at slow current speeds of 2 cm $s^{-1}$ a population of 1000

mussels m$^{-2}$ were capable of capturing approximately 3.5 % of the fish particles available in the horizontal flux. However, when the speed increases to 4 and 8 cm s$^{-1}$, mussels reduced their capture efficiency to 1.7 and 0.9 %, respectively (Cranford et al., 2013). It is possible that the fast currents at the raft close to the fish cages (2.5-13 cm s$^{-1}$) effectively dispersed the particles released by the fish farm; reducing the time they were available to be captured by the mussels' ctenidium. In fact, Cranford et al. (2013) estimated that a capture efficiency exceeding 50% will be only possible at low current speeds (e.g. 2 cm s$^{-1}$). However, an IMTA system with very low hydrodynamic action could negatively influence the renewal of water and natural seston for the filter-feeders, as well as simultaneously increase the accumulation of fish feed and fish and mussel feces beneath the culture units.

The sea bream net-pens studied operated at a relatively small commercial scale with a production that is approximately 10 times lower than a standard commercial salmon pen. The annual stocked biomass of sea bream (450 tons) was lower than reported in other studies which detected greater growth and assimilation of fish feed by integrated mussels (Gao et al., 2006; Handå et al., 2012a). The culture of a low amount of red sea bream biomass and hence, utilization of low amount of feed pellets, could also be a major factor contributing for the lack of enhancement in SFG of the co-cultured mussels. Another factor that may explain the lack of augmented SFG is the availability of feed fines. The manual feeding of fish reduces the abrasion of the pellets caused by pneumatic pipes. As a result, mostly full feed pellets will end up entering the water and much of the feed fine production may remain inside the feed bag. In addition, heat extruded pellets are not disaggregated within the first minutes upon contacting the water.

Furthermore, the high values found for the O:N ratio in mussels held close to the fish cages – indicative of carbohydrate catabolism– and the positive SFG found during all the seasons excepting for the unusually hot summer, pointed out that mussels had sufficient food and energy stores at all times of the year. Hence, a positive effect of particulate organic fish wastes on mussels SFG may only occur in ecosystems that have scarce levels of phytoplankton and/or high inorganic content in the seston (low quality) at least during part of the year (i.e. winter and

autumn) (Troell and Norberg, 1998; Lander et al., 2012; Handå et al., 2012a; Cranford et al., 2013).


## 5. Conclusions

The overall SFG measured in this study was positive and best explained by variations in chlorophyll and quality of the seston during the upwelling–downwelling events. Episodes of negative SFG were detected during the summer stratification, when abnormally high temperatures and low chlorophyll levels resulted in high metabolic expenditure and low energy acquisition. Results suggested that the nanophytoplankton may be the most important size-class for the mussels' diet. This study found no significant differences in the concentrations of chlorophyll and phytoplankton size-classes among both rafts. The results demonstrated that bivalves cultured in the proximity to fish cages did not increase the Scope for Growth and net growth efficiency compared with shellfish held at a reference site. These similar physiological rates were explained by the comparable environmental conditions (chlorophyll, TPM, POM, PIM) measured at both raft stations. The successful utilization of fish particulate wastes was mainly restricted by the placement of the mussels too distant from the fish cages combined with the action of fast currents that seemed to dilute and disperse any wastes too quickly to increase the SFG of mussels held close to the cages. Therefore, the meaningful distance between the fish and bivalves' culture facilities, joined with the energetic hydrodynamism, low feed utilization and non-limiting seston levels were the main reasons for the lack of enhanced SFG of the mussels cultured in the proximity to the fish cages. This study demonstrated that the SFG is a useful model to assess the energetic status of mussels cultured in the proximity of fish cages and it could be further utilized to evaluate the potential of establishing open-water IMTA systems. A significant increase in the SFG of mussels cultured in proximity to fish cages would reflect the effective utilization of fish particles by the shellfish and support the implementation of fish and mussel integrated farming.

# Chapter 4

## Growth variations within a farm of mussel (*Mytilus galloprovincialis*) held near fish cages: importance for the implementation of integrated Aquaculture

## ABSTRACT

Fish farming releases extensive amounts of particulate organic waste that can be exploited by bivalves in integrated culture. We tested if mussels *Mytilus galloprovincialis* cultured at two depths (1 and 6 m) in a raft moored 170 m from a fish farm had greater growth than bivalves held 550 m from the fish cages. Mussel growth was monitored monthly covering the second phase of the culture, from thinning-out to harvest (March to November 2011). We also studied if fish solid and dissolved nutrients increased the organic content of the seston and chlorophyll-*a* levels near the fish cages through weekly samples. Results showed no differences in seston, chlorophyll and physiochemical characteristics of the water among rafts. Maximum growth and Condition Index (CI) occurred during spring-summer (April-August), when mussels had access to greater food quality and quantity. Mussels cultivated close to the cages showed similar shell length, weight and CI compared with mussels distant from the fish farm. Average shell length, meat dry weight and CI at harvest were 76.31 mm, 2.51 g and 23 %. Bivalves cultured distant from the fish cages displayed 26 % higher biomass than the other raft at the end of the experiment. Differences in biomass were explained by the significantly higher recruitment of mussel seed observed at the raft distant from the fish cages from June to November. The lack of a significant enhancement in growth of the bivalves cultured next to finfish is discussed.

# 1. Introduction

Marine fish-farming practices discharge extensive amounts of nutrient rich effluents that need to be treated with cost-effective methods. Fish are cultured in floating net cages with a constant renewal of water that disperses the particulate and dissolved nutrient loading to the surrounding environment. The simultaneous cultivation of finfish with lower trophic level organisms like bivalve molluscs or algae can reduce the impact and amount of fish waste while diversifying and increasing the economic profitability of the production (Troell et al., 2003). This practice is known as Integrated Multi-Trophic Aquaculture (IMTA) and has its origins in freshwater pond polyculture systems in Asia. IMTA is an ecosystem-based approach that aims to recycle the energy contained in the waste of fish through feeding other organisms that transform the effluent into harvestable biomass (Troell et al., 2003; 2009; Neori et al., 2004; 2007).

The culture of bivalve molluscs in close proximity to fish cages has been proven beneficial in several studies. Jones and Iwama (1991) and Jiang et al. (2013) found greater Condition Index (CI), increased shell length and weight in oysters *Crassostrea gigas* held near a fish farm. Several other experiments reported enhanced growth rates and CI in mussels *Mytilus galloprovincialis* (Peharda et al., 2007; Sarà et al., 2009; 2012) and *Mytilus edulis* (Lander et al., 2012; Handå et al., 2012a) cultured close to fish cages. Increments in growth and meat yield in co-cultured bivalves have been attributed to increases in organic particles and phytoplankton concentrations in the surroundings of the fish farm. Thus, bivalves in IMTA systems can benefit directly from uneaten feed pellets and fish faeces organic particles (Mazzola and Sarà, 2001; Reid et al., 2010; MacDonald et al., 2011), or indirectly through the enhancement of primary productivity (Sarà, 2007; Pitta et al., 2009). Nevertheless, results from other studies have not encouraged integrating bivalves with fish as they found none (Taylor et al., 1992; Cheshuk et al., 2003; Navarrete-Mier et al., 2010) or little (Stirling and Okumus, 1995) increase in mussels' growth and CI when cultured close to fish cages. These results have generated a controversy regarding the success with which bivalves can utilize the uneaten fish pellets and feces in open-

water IMTA and thus, there is still a need to continue researching the possibility of the integrated culture under different environmental conditions and husbandry practices.

Mussel farming in Galicia is performed in rafts of 550 m$^2$ built with eucalyptus timber beams supported by 4-6 floats. A maximum of 500 nylon ropes of 12 m length are hung from each raft (Labarta et al., 2004). During the culture process, one or more reductions in rope density (thinning-out) can be performed to avoid crowding conditions (Cubillo et al., 2012). The production cycle lasts 16-18 months and consists of three different stages: obtaining and growing the seed, thinning-out the ropes and harvesting the mussels (Pérez-Camacho et al., 2013). In this work, mussel growth was studied during the second phase of the culture, from thinning-out to harvest (March-November). We focused on a commercial mussel farm placed in the proximity of fish aquaculture facilities situated in the Ría Ares-Betanzos (Galicia, NW Spain). The main objective was to assess if the growth, Condition Index and biomass of cultured mussels (*Mytilus galloprovincialis*) close to the fish farm were enhanced in comparison with those cultured distant from the fish cages in the same mussel farm.

## 2. Materials and methods

### 2.1. Study area

The field survey was performed in Lorbé raft polygon, located on the southern side of the Ría Ares-Betanzos (NW Iberian Peninsula; 43°23'24.74''N; 8°17'48.30''W). To test whether caged fish effluents might enhance mussel growth, bivalves were sampled at two commercial rafts: P-46 and P-14 (Fig. 1). Raft P-46 was located in the outer region of Lorbé, moored at 16 m depth and placed 550 m north from a commercial farm of red sea bream (*Pagellus bogaraveo*). The raft P-14 was placed in the inner region of the polygon anchored at 14 m depth and placed 170 m away from the fish cages.
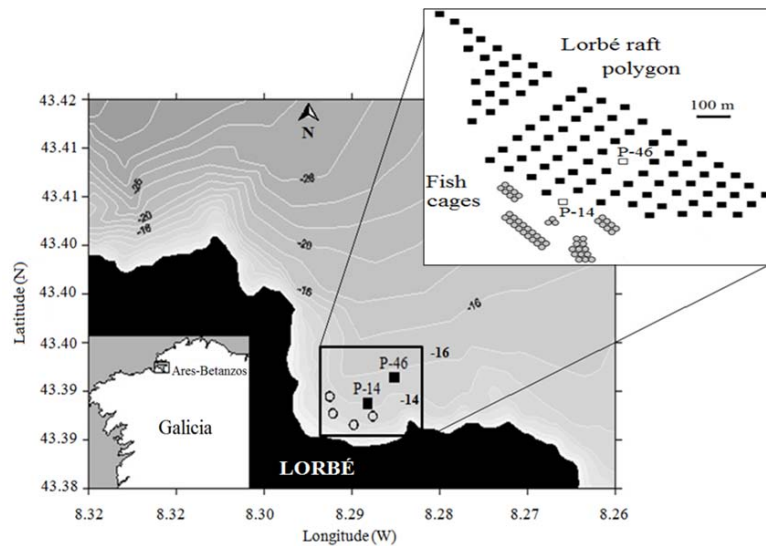
Fig. 1. Map of the experimental site (Lorbé) located in Ría of Ares-Betanzos (Galicia, NW Spain). Open circles indicate the position of the fish cages and squares indicate the position of the mussel rafts.

## 2.2. Sampling schedule and experimental design

The experiment started in March 2011 with the thinning-out of the ropes to obtain a more homogeneous size distribution. New ropes were prepared with a density of about 800 mussels m$^{-1}$. Initial mean shell length and (SD) was 28.57 (0.56) mm and average total dry weight (TDW), tissue (DWt) and shell DW (DWs) were 0.58 (0.02), 0.071 (0.00) and 0.51 (0.01) g mussel $^{-1}$, respectively.

Triplicate water samples of 1 l were collected every week from March to November at 1 and 6 m depth to analyze the concentration of chlorophyll-$a$ (chl-$a$, µg l$^{-1}$) and the characteristics of the seston (see Filgueira et al., 2009 for detailed methodology). We determined the particulate organic matter (POM, mg l$^{-1}$), the inorganic matter (PIM, mg l$^{-1}$) by weight loss after ignition and total particulate matter (TPM, mg l$^{-1}$) as the sum of both fractions. Seston quality ($f$) was computed as the organic fraction contained in the total particulates ($f$=POM/TPM). Physicochemical parameters were weekly monitored with a multiparameter probe YSI 556 to

determine the temperature of the water (T, ºC), salinity (S, ‰), pH and oxygen ($O_2$, mg $l^{-1}$) at both locations and depths.

Mussel growth was monitored during 9 monthly samples (March to November 2011) at rafts P-46 and P-14, including the period from thinning-out to harvest. In each sampling, two ropes per raft were lifted using a hydraulic crane and two replicate samples were collected at 1 and 6 m depth, respectively. Mussels were detached from an estimated rope length of 100 cm containing between 250-400 individuals per replicate. Mussels were counted and weighed to estimate the density (individuals $m^{-1}$ rope) and wet weight (kg $m^{-1}$ rope). Settlement of mussel seed was observed from June to the end of the culture, so seed density and wet weight were also calculated. Total biomass yield (kg $rope^{-1}$) was calculated with a digital dynamometer (0.1 kg precision). Nine ropes per raft were lifted every month to obtain the weight of the ropes in the water (PW). We estimated the biomass using the equation: Biomass = 4.966 PW + 7.011 ($R^2$ = 0.9896), according to Pérez-Camacho et al. (2013).

Mussel shell length (L, mm) was measured as the maximum anterior-posterior axis to the nearest 0.1 mm, using vernier calipers (Mitutoyo®). Mussels were pooled into 1 mm size classes to calculate the adjusted shell length ($L_a$): $L_a = \Sigma (C_L F_L) / N$, where $C_L$ is the individual length class, $F_L$ is the frequency of individuals belonging to each class, and N is the total number of individuals. Total, tissue and shell dry weights were obtained for each replicate from subsamples of 15-20 mussels lying within the interquartile range. Each mussel was dissected and the flesh was separated from the shell and dried at 110 °C until constant weight to obtain DWt and DWs. The TDW was computed as the sum of the DWt and DWs. Regression models plotting the log-transformed TDW, DWt and DWs against log-transformed length were calculated to obtain the weight values corresponding to the adjusted shell length ($L_a$), using the equation: *log DW = log a + b log L.* The Condition Index (CI) was calculated following the equation proposed by Freeman (1974): *CI = (tissue dry weight / shell dry weight) x 100.*

## 2.3. Growth curves and growth rates

Gompertz growth models were fitted to the shell length and weight monthly data. The Gompertz model is a sigmoidal growth curve that predicts a decrease of growth with size or weight. The general form of the model is: $Y_t = Y_\infty (e^{-e(-k(t-t'))})$, where $Y_t$ is the length (mm) or weight (g) at time t (days), $Y_\infty$ is the maximum length or weight, k is the growth parameter that indicates the speed at which maximum value of the dependent variable is obtained (days$^{-1}$) and t' is the inflexion point of the curve, where growth is no longer linear (Ratkoskwy, 1990). The parameters were estimated by non-linear regression, following the Levenberg-Marquardt algorithm and least squares as loss function. The differences between the estimated parameters obtained for each raft and culture depth were analyzed using an extra sum-of-squares F-test (Motulsky and Christopoulos, 2004): *F=((RSSs)− (RSSi)) / ((dfs−dfi) / (RSSi/dfi))* where RSSs and RSSi are the residual sum of squares of the curves fitted with and without a parameter shared, respectively, and dfs and dfi are their corresponding degrees of freedom.

Growth rates (GR) corresponding to the length (mm day$^{-1}$) and weight (g day$^{-1}$) for the spring-summer period (April-August), the summer-autumn period (August-November) and the complete experimental period (April-November), were calculated as the difference between the biometric values at the end and the beginning of each period.

## 2.4. Total biomass yield

Generalized additive models (GAM) were fitted to explore the temporal variation of the mussels' biomass, with smoothing functions for month, in order to compare the changes within the two rafts. The relationship between the mean of the dependent or response variable (E (Y)) and an *m* pool of independent variables (*X*) adopted the following GAM function:

$$E(Y) = c + \sum_{j=1}^{m} S_j(x_j) + \varepsilon$$

The model had an intercept ($c$) and a non-parametric component that integrated the sum of the unknown smoothing functions ($S_j$) of the explanatory variable month ($x_j$) and an error $\varepsilon$. The smoothing functions were represented as a linear combination of splines functions.

Shapiro-Wilk test was used to detect normality of distribution of the response variable. In the case the dependent variable followed a Gaussian family distribution ($p > 0.05$) we established an identity link. When the dependent variable did not fit normality we set a Gamma family distribution and a logarithmic link function.

## 2.5. Statistical analyses

Environmental and physicochemical parameters, shell length, weight measurements, CI, density and growth rates were analyzed with a two-way analysis of variance (ANOVA) followed by Tukey's HSD to test for differences between rafts and culture depths during the 9 months of the culture. The assumptions of normality and homogeneity of variances were previously tested with Shapiro-Wilk and Levene test, respectively.

Size frequency distributions were analyzed at the beginning (March) and the end (November) of the experimental culture with an ANOVA on ranks, as normality and homogeneity of variances were not met. Wilcoxon ranked-sum test for two independent samples was used as a *post-hoc* test to compare the size frequency distributions between rafts P-46 and P-14 at the two experimental depths. Statistical analyses were performed using Statistica version 7.0 (StatSoft Inc). All the GAM modeling analyses were done with the software R-project, using the packages *mgcv* and *splines*, available from the Comprehensive R Archive Network (CRAN).

# 3. Results

## 3.1. Environmental and physicochemical characteristics

The total particulate matter (TPM), organic (POM) and inorganic (PIM) fractions of the seston had average concentrations of $0.84 \pm 0.60$ mg $l^{-1}$, $0.50 \pm 0.49$ mg $l^{-1}$ and $0.34 \pm 0.38$ mg $l^{-1}$ (Fig. 2 A). Average seston quality (*f* ) was $0.62 \pm 0.22$, whereas mean chlorophyll-*a* values were $1.78 \pm 1.68$ µg $l^{-1}$. No significant particulate matter, seston quality and chlorophyll differences were found between locations and depths during the complete sampling period (Tukey HSD; p > 0.05).

Temperature and salinity averaged $15.45 \pm 1.67$ ºC and $35.82 \pm 0.55$ ‰. The pH and $O_2$ of the water averaged $8.01 \pm 0.31$ and $8.39 \pm 0.91$ mg $l^{-1}$ (Fig. 2 B). The two-way ANOVA followed by *post-hoc* testing indicated that from April to August (spring-summer) temperature was significantly higher at 1 m depth than at 6 m (Tukey HSD; $P < 0.05$). Similarly, salinity was higher at 1 m than at 6 m from March-August. ANOVA showed no differences in temperature and salinity among the raft stations ($P > 0.05$). No significant pH and $O_2$ differences were found between rafts and depths during the entire experimental period (Tukey HSD; p > 0.05).

A)
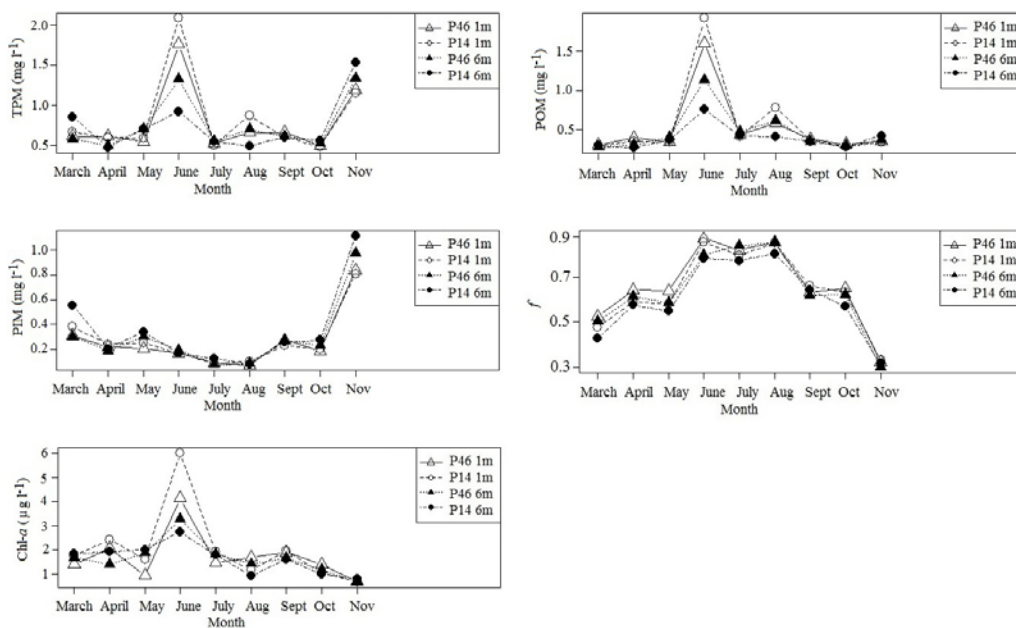
B)
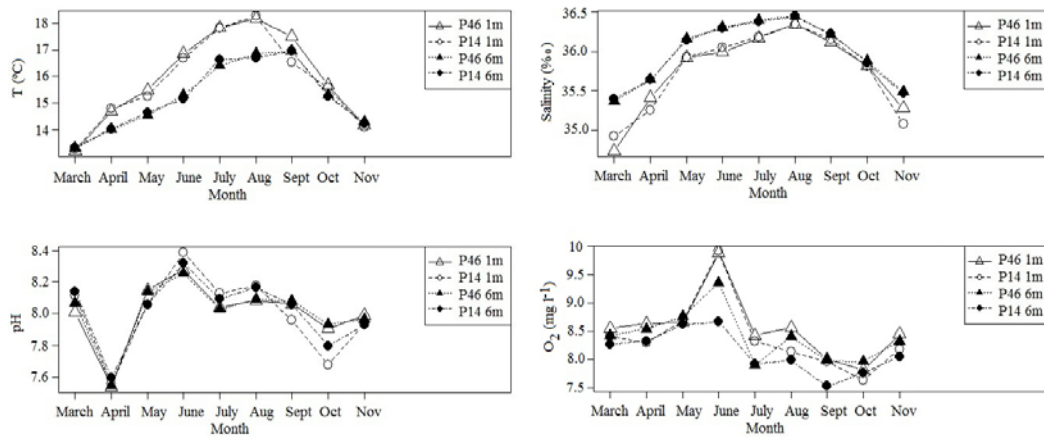


Fig. 2. Average values obtained for: A) the characteristics of the seston (total particulate matter, organic and inorganic matter), seston quality ($f$=POM/TPM) and chlorophyll-$a$; B) physicochemical characteristics of the water during the complete culture period at the rafts distant (P-46) and close (P-14) to the net-pens at 1 and 6 m depth. Each monthly data point is based on the average of four weekly samples.

## 3.2. Mussel growth and Condition Index (CI)

No significant differences in the shell length (L), tissue (DWt), shell (DWs) and total dry weight (TDW) were found between rafts and depths at the beginning of the culture (March) (Tukey HSD; p>0.05), ensuring that mussels at both sites started at similar length and weight conditions (Table 1). Mussels' main growth was observed from May to September, after which shellfish entered a steady state until being harvested in November (Fig. 3). Mussels reared at the raft far from the fish cages grew 48.34 mm and gained 2.62 g DWt, 10.52 g DWs and 13.14 g TDW after 9 months of cultivation. On the other hand, mussels cultured close to the fish farm gained 47.14 mm, 2.27 g DWt, 10.33 g DWs and 12.61 g TDW (Table 1). At harvest, significantly greater length and weight values were found for mussels cultured distant from the fish farm at 6 m, followed by those cultured closer to the fish cages at 1m depth (Tukey HSD; p<0.05; Table 1).
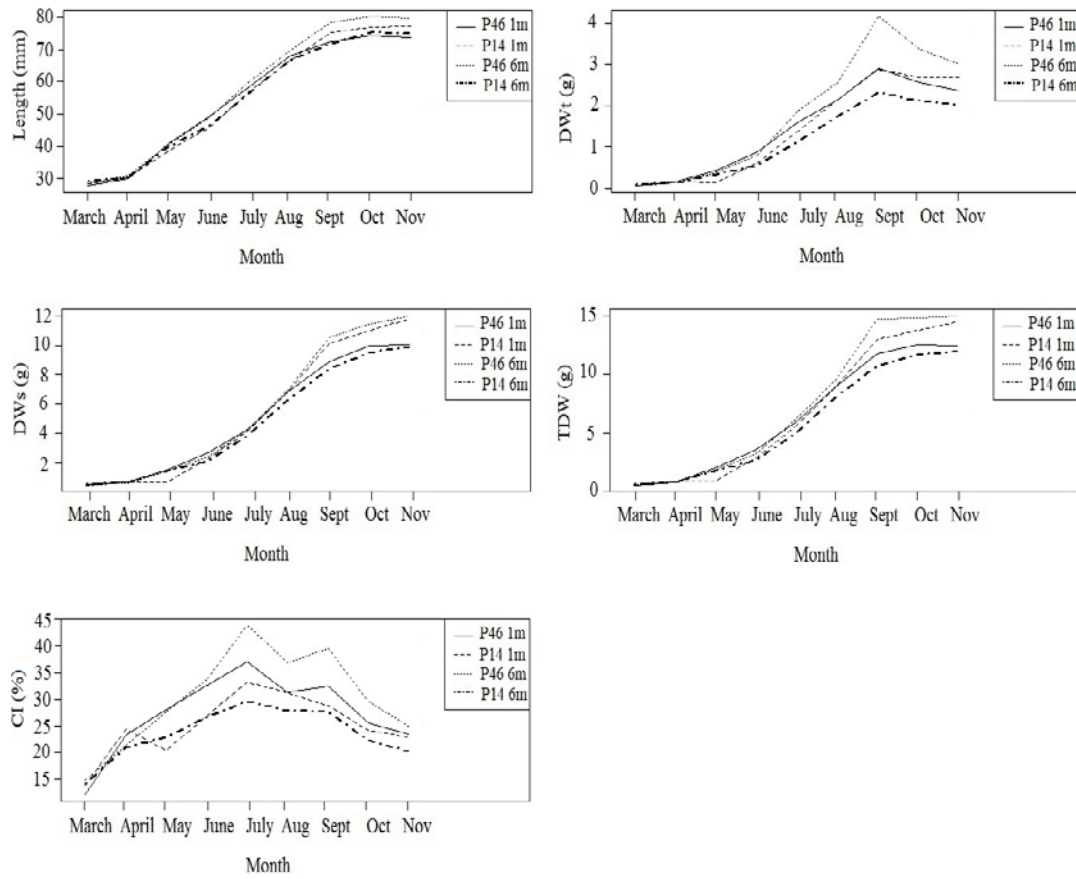
Fig. 3. Monthly mean shell length (L, mm), tissue dry weight (DWt, g), shell dry weight (DWs, g), total dry weight (TDW, g) and Condition Index (CI, %) values registered for mussels sampled at the rafts distant (P-46) and close (P-14) to the fish farm at 1 and 6 m depth.

| Month | Shell length | | | | Dry Weight Tissue | | | | Dry Weight Shell | | | | Total Dry Weight | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 m | | 6 m | | 1 m | | 6 m | | 1 m | | 6 m | | 1 m | | 6 m | |
| | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 |
| March | 27.8 (0)a | 28.6(0)a | 28.56(0)a | 29.2(0)a | 0.06 (0)a | 0.06(0)a | 0.07(0)a | 0.077(0)a | 0.5(0)a | 0.4(0)a | 0.5(0)a | 0.5(0)a | 0.5(0)a | 0.5(0)a | 0.5(0)a | 0.6(0)a |
| November | 73.6(2.7)a | 77.2(0.9)b | 79.5(1.9)b | 74.9(1.1)a | 2.3(0.2)a | 2.6(0)b | 2.9(0.1)c | 2.0(0)a | 10.0(0.6)a | 11.7(0.2)b | 11.9(0.6)b | 9.9(0.3)a | 12.4(0.9)a | 14.4(0.3)b | 14.9(0.8)b | 11.9(0.3)a |

Table 1. Average values and standard deviation (SD) obtained for the shell length (mm) and tissue, shell and total dry weight (g) during the beginning (March) and end (November) of the culture period for the rafts distant (P-46) and close (P-14) to the net-pens, at both experimental depths. The different letters indicate significant differences (Tukey HSD; $P < 0.05$).

| Periods | GR Shell length | | | | GR Dry Weight Tissue | | | | GR Dry Weight Shell | | | | GR Total Dry Weight | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 m | | 6 m | | 1 m | | 6 m | | 1 m | | 6 m | | 1 m | | 6 m | |
| | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 |
| Spring-Summer | 0.30(0)a | 0.28(0)b | 0.31(0)c | 0.29(0)b | 0.01(0)a | 0.01(0)a | 0.02(0)b | 0.01(0)a | 0.05(0)a | 0.04(0)b | 0.05(0)c | 0.04(0)b | 0.06(0)a | 0.06(0)a | 0.07(0)b | 0.05(0)a |
| Summer-Autumn | 0.06(0.02)a | 0.09(0.01)a | 0.08(0.02)a | 0.07(0.01)a | 0.002(0)a | 0.005(0)b | 0.004(0)b | 0.002(0)a | 0.02(0)a | 0.04(0)b | 0.04(0)b | 0.03(0)a | 0.03(0)a | 0.04(0)b | 0.04(0)b | 0.03(0)a |
| Spring-Autumn | 0.21(0.01)a | 0.22(0)a | 0.23(0)b | 0.21(0.01)a | 0.011(0)a | 0.01(0)a | 0.01(0)b | 0.009(0)a | 0.04(0.)a | 0.05(0)b | 0.05(0)b | 0.04(0)a | 0.05(0)a | 0.06(0)b | 0.06(0)b | 0.05(0)a |

Table 2. Average values and standard deviation (SD) obtained for the growth rates (GR) in terms of shell length (mm day$^{-1}$) and tissue, shell and total dry weight (g day$^{-1}$) during the Spring-Summer (April-August), Summer-Autumn (August-November) and complete culture period for the rafts distant (P-46) and close (P-14) to the fish farm at both experimental depths. The different letters indicate significant differences (Tukey HSD; $P < 0.05$).

Mussels' Condition Index (CI) increased during spring and summer (March-August) and decreased thereafter until harvest (Fig. 3). Maximum Condition Index was observed in July at the raft distant (43.93 %) and close (33.71 %) to the fish net-pens (Fig. 3). Results indicated that during May, June and October, mussels distant from the fish farm had significantly greater CI at both culture depths than the other raft. During the months of July, August and November, mussels distant from the fish cages at 6 m had significantly greater CI than the other raft at both depths (Tukey HSD; $P < 0.05$).

## 3. Growth rates (GR)

Growth rates (GR) were analyzed segregating the experimental time into three periods: spring-summer (April-August), summer-autumn (August-November) and the entire experimental period (April-November) (Table 2).

The greatest growth rates were observed during the spring-summer period. During this period, significantly greater GR in L, DWt, DWs and TDW were observed at the raft distant from the fish farm at 6 m depth compared to the other raft at both depths (Tukey HSD; $P < 0.05$; Table 2). During the summer-autumn period higher GR in DWt, DWs and TDW were obtained for P-46 at 6 m and P-14 at 1m (Table 2). Regarding the entire experimental period, results highlighted significantly higher GR in length in mussels distant from the fish cages at 6 m, and higher GR in weight were found in P-46 at 6 m and P-14 at 1 m (Tukey HSD; $P < 0.05$; Table 2).

## 3.4. Mussel density and wet weight

In general, live mussel density and wet weight were similar at both rafts and depths during the entire experimental culture (Tukey HSD; $P < 0.05$; Table 3). The lower mussel density and weight observed at 1m during the last culture month (November) was attributed to mortality and dislodgements from the ropes due to the growth of recruited seed. Mussel seed settlement was

observed on the ropes from June to November at both rafts and depths, although in P-14 at 6 m it was negligible. Seed density and weight were greater at 1 m depth than at 6 m, and values obtained at the raft distant from the fish cages doubled the amount obtained at the other raft (Tukey HSD; $P < 0.05$; Table 4). Thus, the greatest seed recruitment was observed in the raft P-46 at 1 m and seed settlement decreased with depth at both sites.

| | Mussel wet weight (kg m$^{-1}$) | | | | Mussel Density (indiv m$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| | 1m | | 6 m | | 1m | | 6 m | |
| Month | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 |
| June | 3.8(0.5)a | 3.7(1.2)a | 3.8(0.1)a | 3.9(0.18)a | 272(4.9)a | 294(41.7)a | 307(43.1)a | 401(17.6)b |
| July | 7.2(0.9)a | 6.2(0.3)a | 6.3(0)a | 6.3(0.4)a | 357(24)a | 330(50.2)a | 309(4.2)a | 387(16.9)a |
| August | 8.5(0.8)a | 9.2(0.1)a | 8.9(0.5)a | 7.5(0.1)a | 263(21.2)a | 325(8.5)b | 295(9.2)a | 291(5.6)a |
| September | 10.7(0.6)a | 13.6(1.3)a | 12.68(1.9)a | 10.6(2.5)a | 239(14.8)a | 320(6.3)a | 299(48.7)a | 331(99.7)a |
| October | 11.7(0.2)a | 13.1(1.8)a | 14.4(1.3)a | 13.9(3.3)a | 246(11.4)a | 265(20.5)a | 326(36)a | 292(74.2)a |
| November | 6.9(1.5)a | 6.4(0.5)a | 10.3(4.1)a | 11.8(4.1)a | 161(23.2)a | 137(10.6)a | 218(82.9)a | 335(138.2)a |

Table 3. Average values and standard deviation (SD) obtained for the mussel wet weight (kg m$^{-1}$ rope) and density (individuals m$^{-1}$ rope) from June to November for the rafts distant (P-46) and close (P-14) to the net-pens, at both experimental depths. The different letters indicate significant differences (Tukey HSD; $P < 0.05$).

### 3.5. Size frequency distribution

The unilateral Wilcoxon test revealed that there were no differences in the shell size (mm) frequency distributions among both rafts and depths at the beginning of the culture (p > 0.05; Fig. 4A). However, the unilateral Wilcoxon test for November (harvest) showed that mussels reared on the raft distant from the fish cages (P-46) at 6 m reached significantly greater sizes than mussels harvested at 1 m within the same raft and at 6 m on the other raft (Table 5; Fig. 4B). Furthermore, mussels sampled at 1m in the raft close to the fish cages (P-14) had greater

shell length than mussels at 6 m within the same raft and those reared at the same depth distant

from the farm (P-46) (Table 5; Fig. 4B).

| | Seed wet weight (kg m$^{-1}$) | | | | Seed density (indiv m$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 m | | 6 m | | 1 m | | 6 m | |
| Month | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 |
| June | 0.007(0)a | 0.0008(0)a | 0 a | 0 a | 611(64.6)a | 335(244.6)b | 0 c | 0 c |
| July | 0.2(0)a | 0.1(0)a | 0.001(0)c | 0 c | 6098(3437.9)a | 3162(1992.6)b | 13(5.6) c | 0 c |
| August | 2.2(0.4)a | 0.8(0.1)b | 0.2(0.1)c | 0 c | 3607(1030.9)a | 2570(610.9)b | 198(123)c | 0 c |
| September | 11.4(0.4)a | 2.3(0.1)b | 0.5(0)c | 0 c | 4649(636.3)a | 891(199.4)b | 144(51.6)c | 0 c |
| October | 9.7(0.1)a | 4.8(1.7)b | 0.5(0)c | 0.05(0)c | 2730(228.0)a | 1536(713.0)b | 35(5)c | 21(14.8)c |
| November | 6.7(2.1)a | 2.5(1)b | 3.5(0)c | 0.08(0)c | 1687(470.2)a | 815(357.0)b | 786(75.8)b | 12(7)c |

Table 4. Average values and standard deviation (SD) obtained for the seed wet weight (kg m$^{-1}$ rope) and seed density (individuals m$^{-1}$ rope) from June (first seed recruitment detected) to November for the rafts distant (P-46) and close (P-14) to the net-pens, at both experimental depths. The different letters indicate significant differences (Tukey HSD; $P < 0.05$).
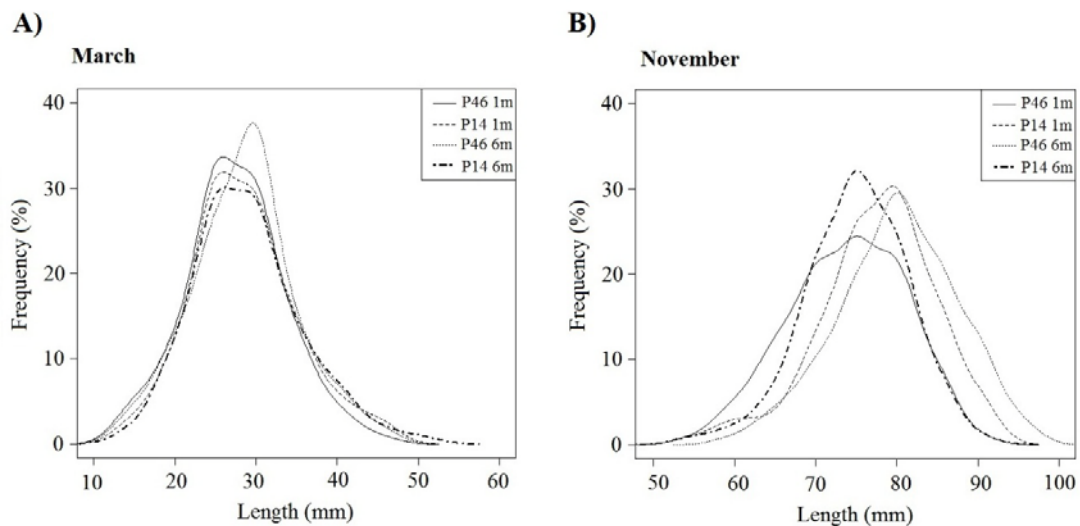


Fig. 4. Shell size frequency distributions A) at the beginning of the culture (March) and B) harvest (November) obtained for the mussels cultured at the raft distant (P-46) and close (P-14) to the fish farm at both experimental depths.

### 3.6. Gompertz growth curves

No significant differences were observed for the estimated asymptotic shell length ($L_\infty$) between culture sites or experimental depths (F test; $P > 0.05$; Table 6; Fig. 5). Moreover, no significant differences were obtained for the growth factor (k) and the inflexion point of the shell length growth curve (t') between depths at both culture sites (F test; $P > 0.05$).

Significant differences were observed for the asymptotic tissue dry weight values ($DWt_\infty$) between both rafts and depths. Mussels cultured distant from the farm at 6 m depth had a $DWt_\infty$ 37.07 % larger than mussels at raft P-14 at 6 m (Table 6; Fig. 5). Mussels at P-14 at 1m depth reached greater tissue weight than P-46 at the same depth (5.80 %), and the asymptotic growth was reached later (Table 6; Fig. 5).

Shellfish reared at P-46 at 6 m achieved 17.33 % and 19.84 % greater $DWs_\infty$ and $TDW_\infty$ than those at the other raft. Mussels held close to the fish farm at 1 m reached 12.58 % and 11.56 % greater $DWs_\infty$ and $TDW_\infty$ than mussels at raft P-46 at 1 m (Table 6; Fig. 5). There were significant differences in the growth factor of the DWs and TDW between rafts at both depths. DWs and TDW growth curves changed earlier from linear to asymptotic in P-46 at 1 m, and in P-14 at 6 m (Table 6).

|          | P-46 1m | P-14 1m | P-46 6m | P-14 6 m |
|----------|---------|---------|---------|----------|
| P-46 1m  | -       | $4.11\,e^{-10}$ *** | $2.2\,e^{-16}$ *** | 0.04 ** |
| P-14 1m  | 1       | -       | $1.33\,e^{-06}$ *** | 1 |
| P-46 6 m | 1       | 1       | -       | 1 |
| P-14 6 m | -       | $2.62\,e^{-10}$ *** | $2.2\,e^{-16}$ *** | - |

Table 5. Results for the Wilcoxon rank-sum test for two independent samples comparing the size frequency distributions between the rafts distant (P-46) and close (P-14) to the fish cages at both experimental depths (1 and 6 m) at the end of the experiment in November ($H_0$: $L_{row} > L_{column}$). Significant differences are denoted by ** $P < 0.01$, *** $P < 0.001$.
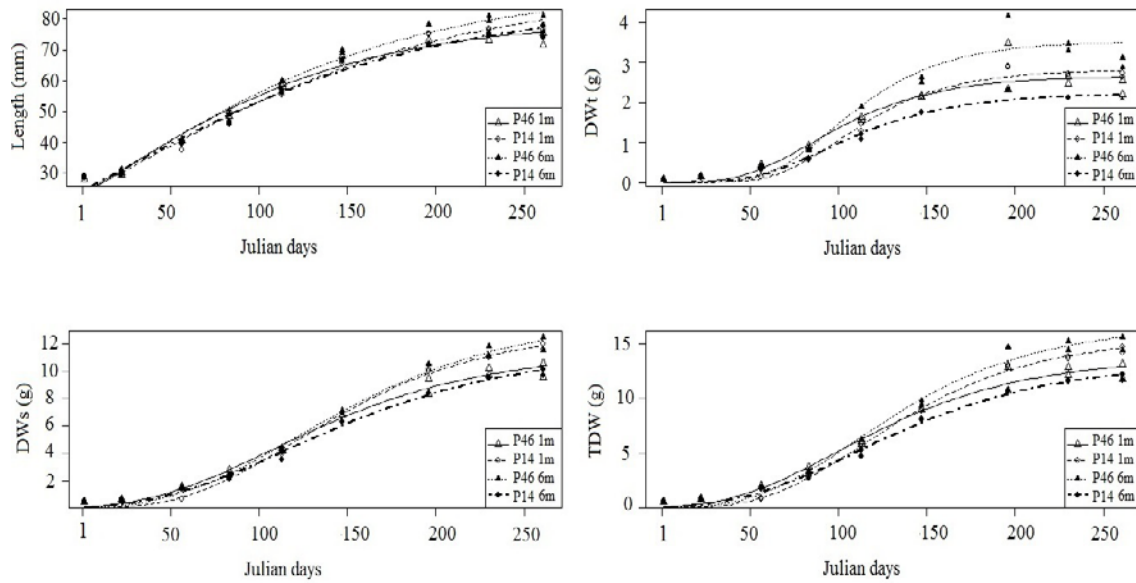
Fig. 5. Growth curves fitted to the Gompertz model for shell length, tissue dry weight, shell dry weight and total dry weight for both rafts (P-46 and P-14) and depths (1 and 6 m).

## 3.7. Biomass yield

A General Additive Model (GAM) was established to study spatial and temporal variations in mussels' biomass (kg rope$^{-1}$) during the 9 months of the experimental culture:

*Biomass P-46 = 85.534 + S $_{P-46}$*

*Biomass P-14 = 75.680 + S $_{P-14}$*

Where *S* denotes the unknown smoothing functions of culture time for the biomass of each raft. The models presented a good fit with determination coefficients of $R^2 = 0.994$ and $R^2 = 0.996$ for raft P-46 and P-14, respectively (Fig. 6). The biomass production increased 9-fold from the thinning-out of the ropes in March to harvest in November. Biomass increased rapidly from June to September, after which it started to slow down until harvest (Fig. 6). The average increase in biomass observed from April to August (spring-summer) was 188.32 and 137.31 kg rope$^{-1}$ at the raft distant (P-46) and adjacent (P-14) to the fish cages, respectively. An increase of 154.94 and 104.15 kg rope$^{-1}$ was registered at P-46 and P-14 from the end of the summer until harvest, respectively. On average, the estimated biomass at harvest was 26 % higher at the raft distant from the fish farm (379 kg rope$^{-1}$) than at P-14 (280 kg rope$^{-1}$).

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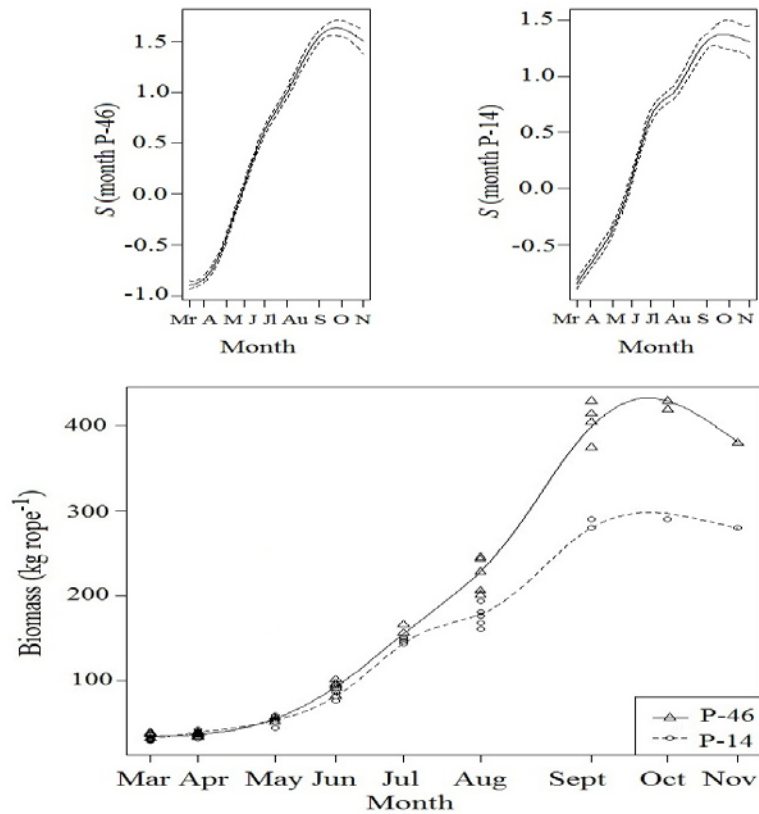
Fig. 6. Generalized Additive Model (GAM) smoothing functions and functional relationships between mussel biomass (kg rope$^{-1}$) and culture time obtained for both rafts.

# 4. Discussion

## 4.1. Temporal variations in mussel growth

This study confirmed that mussels in suspended culture experienced the greatest growth rates during the spring-summer period, coinciding with the upwelling months, when filter-feeders have access to high quality food due to increased production of phytoplankton (Figueiras et al., 2002; Peteiro et al., 2011). Concurrently, decreases in food availability and organic content of the seston observed during the beginning of the downwelling season in late summer and autumn were reflected in a reduction of the bivalves' growth rates. This was in agreement with the growth pattern found by Cubillo et al. (2012) in the same area, who observed that the highest increment in shell length, TDW and DWs of *M. galloprovincialis* was between May-September,

| Parameters | Shell length | | | | Dry Weight Tissue | | | | Dry Weight Shell | | | | Total Dry Weight | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 m | | 6 m | | 1 m | | 6 m | | 1 m | | 6 m | | 1m | | 6 m | |
| | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 |
| $Y\infty$ | 79.83a | 89.36a | 89.96a | 84.67a | 2.66a | 2.82b | 3.53a | 2.22b | 11.51a | 13.17b | 14.04a | 11.60b | 13.80a | 15.61b | 16.73a | 13.41b |
| K | -0.01a | -0.00a | -0.01a | -0.00a | -0.02a | -0.02a | -0.02a | -0.02a | -0.015a | -0.01b | -0.01a | -0.01b | -0.01a | -0.01b | -0.01a | -0.01b |
| t ' | 17.83a | 30.31a | 27.48a | 22.23a | 84.34a | 99.11b | 95.27a | 92.13a | 106.45a | 119.82b | 120.48a | 115.61b | 98.72a | 113.14b | 109.60a | 107.72b |
| $R^2$ | 0.98 | 0.98 | 0.98 | 0.98 | 0.92 | 0.98 | 0.93 | 0.97 | 0.99 | 0.99 | 0.99 | 0.99 | 0.98 | 0.99 | 0.98 | 0.99 |

Table 6. Estimated parameters and determination coefficients obtained for the shell length (L, mm), tissue (DWt, g), shell (DWs, g) and total dry weight (TDW, g) Gompertz growth curves, for mussels cultured distant (P-46) and close (P-14) to the fish farm at 1 and 6 m depth. All parameters are statistically significant ($p<0.001$) and the different letters indicate significant differences between rafts at both depths ($p<0.05$).

while increments in DWt ceased after August, when chlorophyll levels became low. The maximum TPM, POM and chlorophyll concentrations observed in June likely owed to a persistent phytoplankton bloom of harmful algae *Messodinium rubrum* and *Gonyaulax sp.* observed during the sampling (INTECMAR, 2013). On the other hand, a succession of storms during the month of November caused resuspension events that resulted in a dilution of the organic particles and overall quality of the seston by increments in the inorganic material. Seawater physicochemical parameters showed seasonal variations similar to Cubillo et al. (2012). Reductions in salinity were probably associated with freshwater discharges during the rainy months (spring and autumn), while the photosynthetic activity of the phytoplankton in the summer was reflected in the increased pH and oxygen levels in the seawater.

The mussels' Condition Index (CI) varied as a function of the food availability and reflected the different stages of the reproductive cycle and storage of nutrient reserves (Taylor et al., 1992; Pérez-Camacho et al., 1995; Stirling and Okumus, 1995; Cheshuk et al., 2003). Maximum summer CI values and minimum CI in March and November were likely associated with variations in food quality observed during this period (Figs. 2 and 3). Reductions in Condition Index levels found in April, July and September might correspond with the spawning events recorded between spring and early autumn by Peteiro et al. (2011) in the Ría Ares-Betanzos. The major settlement peak of this study (August-September) also agreed with the results of Peteiro et al. (2011), where settlement was concentrated within the favorable upwelling season. Villalba (1995) described that mussels from the northern Rías (i.e. Lorbé, Ares-Betanzos) had a single spawning event between June and July, whereas mussels from the southern Rías had several spawning events during spring and summer. However, results from this study and those of Peteiro et al. (2011) suggested that the reproductive cycle of mussels in Lorbé matched the pattern of the southern Rías.

Mussel biomass on the ropes increased steadily throughout the experimental culture and reached maximum levels during the beginning of autumn (September-October), according to mussel

growth patterns (Table 3). Observations of increased mussel biomass on the ropes partly owed to the large recruitment of mussel seed detected in August and September. The recruitment of mussel seed on artificial collector ropes can reach average densities up to $13,033 \pm 1,136$ mussels m$^{-1}$ and provides 60 % of the seed employed in the mussel industry (Filgueira et al., 2007; Labarta et al., 2004). Seed density and weight were lower in the raft placed close to the fish cages, in the inner region of Lorbé raft polygon, compared to the raft distant from the cages in the outer region. Similarly, Peteiro et al. (2007a, b; 2011) found higher seed density on artificial collectors placed more seaward, attributing the differences in seed recruitment between sites to the water circulation regime of the Ría, larval dispersion patterns and intra-specific competition. Furthermore, we observed that seed was more abundant at 1 m depth than in deeper sections of the ropes. This recruitment pattern has been previously described in the Ría de Arousa and Ares-Betanzos by Fuentes and Molares (1994) and Peteiro et al. (2011), respectively, who found higher densities on seed collectors suspended from the rafts at shallower waters. The higher concentration of larvae at the surface may offer advantages for larvae offshore displacement during upwelling and onshore transport during upwelling relaxation and/or downwelling (Peteiro et al., 2011 and references therein). The large amount of seed recruited on the raft distant from the cages at 1 m depth increased the biomass of the rope and was the most likely cause of the higher length, weight, Condition Index, and GR in TDW and DWt registered at 6 m for adult mussels in this raft. The lower amount of seed at 6 m allowed for higher GR and Condition Index, as adult mussels had less intraspecific competition for food and space than at 1 m depth. Small mussels have a very high filtration capacity and can outcompete adult mussels and other suspension feeders that try to settle in the ropes (Tenore and González, 1975). The slight but significantly higher DWt$_\infty$, DWs$_\infty$ and TDW$_\infty$ found in mussels grown close to the fish cages at 1 m compared with those at the same depth on the outer site suggests that these differences were also a result of competitive pressure exerted by a greater seed recruitment on the outer raft.

## 4.2. Importance for Integrated Multi-Trophic Aquaculture

The results of this study were in agreement with Cheshuk et al. (2003) who reported no enhancement on the growth of mussel *Mytilus planulatus* when cultured at 70 and 100 m from a salmon farm, compared to mussels at 500 and 1200 m from fish cages. Cheshuk only registered significant differences in the shell length and CI, although these differences were minimal (2 mm in length and 11 % in CI). Similar findings have been reported for co-cultivated mussels *M. edulis* and *M. galloprovincialis* (Taylor et al., 1992; Navarrete-Mier et al., 2010), oyster *Ostrea edulis* (Navarrete-Mier et al., 2010) and sea scallop *Placopecten magellanicus* (Gryska et al., 1996; Parsons et al., 2002). Likewise, Both et al. (2012) found that the dry weight, ash-free dry weight, shell length and Condition Index of the individuals of *M. edulis* were significantly higher when fed with algae rather than fish effluents. In the previous studies, seston and chlorophyll concentrations were not enhanced near the fish cages, so mussels did not have any direct (uneaten fish feed and faeces) or indirect (increased chlorophyll production) benefit near the fish farms (Taylor et al., 1992; Cheshuk et al., 2003). Similarly, the weekly data on the physicochemical characteristics of the water, seston and chlorophyll concentrations of this study revealed similar values among raft stations, regardless of the distance between the culture units and the fish cages.

Our results contrasted with several studies that, as a result of the utilization of fish-derived algal and non-algal POM (i.e. pellets and faeces), measured an enhancement in growth of co-cultured mussels (Peharda et al., 2007; Sarà et al., 2009; 2012; Handå et al., 2012a; Lander et al., 2012). Sarà et al. (2012) and Peharda et al. (2007) also found that *M. galloprovincialis* attained greater asymptotic shell length ($L_{\infty}$) close to fish cages than at control sites, whereas no increments in $L_{\infty}$ were found near the fish cages in this study. Furthermore, other studies with sampling series reported enhanced TPM and POM levels at 5 m from the fish cages (Lander et al., 2013), while Sarà et al. (2012) found significantly higher levels of chlorophyll-*a* 50 m from the fish cages. Several surveys determined that the vast majority of the fish solid particles settle within 50-60 m of the fish cages, depending on the current speed, direction and bottom depth (Cheshuk et al.,

2003; Lander et al., 2013). It is probable that elevations in POM, chlorophyll and subsequently, bivalve growth, were not observed in this study due to a separation greater than 50 m between the raft P-14 and the fish cages (170 m). Cheshuk et al. (2003) not only recommended culturing bivalves close to the cages but also at depths greater than 5 m to maximize the capture of fish waste particles coming from the cages. However, differences in growth performance found among both raft stations and depths in this study could not be attributed to any increase in POM or chlorophyll-a at 6 m depth, but to differences in mussel seed settlement.

The disparity between this study and previous IMTA experiments that found enhanced growth of co-cultured mussels may reside in the different environmental conditions and design of open-water IMTA systems (Troell and Norberg, 1998; Troell et al., 1999, 2011). Fish solid particles and dissolved nutrients may not be available in all IMTA systems depending on the scale of the fish farm, particle size of the waste solids, distance between the fish and bivalve facilities, the response time of the local primary production to the excess nutrients, ambient seston quality and quantity and speed and direction of the current (Cheshuk et al., 2003; Troell and Norberg, 1998; Troell et al., 1999, 2011). Previous studies performed in Lorbé raft polygon revealed that mussels cultured in a raft near the fish cages did not increase their absorption efficiency (Irisarri et al., 2013). The high residual current speed of the raft near the fish cages (average 3.70 cm s$^{-1}$) (Zuñiga et al., 2014), low annual stock of fish biomass (450 tons), high natural availability of food and significant distance between the mussel and fish facilities (170 m) (Irisarri et al., 2013) seem to be the major factors causing the lack of enhancement in growth of the mussels cultured close to the fish cages. The design of the IMTA system also needs to consider the phytoplankton and seston characteristics of the ecosystem, as increased growth and effluent bioremediation by co-cultured bivalves has been more apparent during periods of adverse environmental conditions (i.e. winter and autumn) for mussel growth (Lander et al., 2012; Handå et al., 2012a). The great primary production and high seston quality ($f \geq 0.5$) in the Galician Rías support one of the greatest mussel growth yields in the world (Figueiras et al., 2002) and therefore, it seems plausible that additional organic nutrients released by fish may not represent a significant energetic input for mussels reared in this ecosystem. Although results of this study

demonstrated that mussels cultured close to a fish farm did not increase their growth, experiments that did obtain higher growth rates were performed under environmental and husbandry conditions not comparable with our study. Differences between this study and experiments that found an increment in bivalve growth reflect that site-specific hydrodynamic conditions, primary productivity, seston concentration and culture practices are the main factors that should be considered for the hypothetical design of an open-water integrated culture.
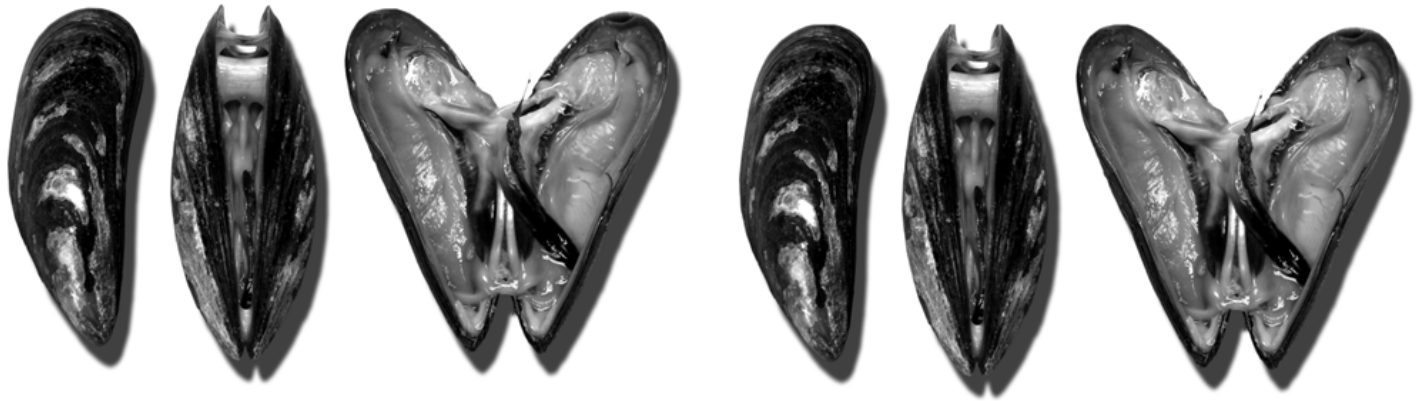
# CHAPTER 5

## FATTY ACIDS AS TRACERS OF TROPHIC INTERACTIONS BETWEEN SESTON, MUSSELS AND BIODEPOSITS IN A COASTAL EMBAYMENT OF MUSSEL RAFTS IN THE PROXIMITY OF FISH CAGES

# ABSTRACT

We traced the food sources of mussel *Mytilus galloprovincialis* cultured in suspension in Ría Ares-Betanzos (N.W. Spain) by means of fatty acid (FA) biomarkers. The FA profile of seston, mussels' mantle, digestive gland and feces was analyzed during five seasons. Due to the proximity of a fish farm to the bivalve aquaculture site, we also tested if mussels and seston situated 170 m distant from the fish cages incorporated fish feed FA markers compared with samples obtained 550 m away. The principal FA in the mussels' organs were 16:0, 16:1ω7, EPA (20:5ω3) and DHA (22:6ω3), while 16:0 predominated in the feces. Seasonal fluctuations in the seston composition were mirrored in the FA signature of mussels' organs and feces, although the digestive gland had the closest resemblance to the seston FA profile. In general, diatom and bacteria derived-biomarkers predominated in mussels' organs and feces during the upwelling period (spring-summer), while dinoflagellates were the dominant dietary source during downwelling (autumn-winter). The higher concentration of EPA and DHA in both organs and the feces compared with the seston suggested a preferential accumulation of these ω3 FA in the mussels' tissues. The results showed a lack of assimilation of fish feed FA biomarkers in the seston and mussel samples. This might be due to the dispersion of uneaten feed particles by high current velocity, substantial distance between the fish and mussel culture, the limited amount of nutrient waste released by the fish farm and dilution of feed particles in the large mussel standing stock.

*Key words: Fatty acid biomarkers; seston; digestive gland; mantle; mussel feces.*

# 1. Introduction

Fatty acids (FA) are valuable biochemical markers to trace the flow of organic matter along different trophic levels in marine food webs (Dalsgaard et al., 2003; Kelly and Scheibling, 2012). Fatty acids provide a qualitative measurement of the energy transferred from primary producers up to higher trophic levels (Dalsgaard et al., 2003) and have the advantage that once stored in the body they don't undergo major changes (Graeve et al., 1994; Xu and Yang, 2007; Kelly and Scheibling, 2012). According to the 'you are what you eat' principle, the FA profile of consumers reflects the composition of their diet and their trophic relationships, even if most taxa lack a fat-storage organ and are capable of modifying their FA composition depending on the environmental characteristics, the physiological status and the turnover rate of each tissue (Ventrella et al., 2008; Kelly and Scheibling, 2012; Richoux et al., 2014). Previous studies have successfully used FA markers to investigate the trophic interactions and feeding ecology of mussels and their food sources (Budge et al., 2001; Alkanani et al., 2007; Shin et al., 2008; Ventrella et al., 2008; Ezgeta-Balić et al., 2012; Najdek et al., 2013; Zhao et al., 2013; Richoux et al., 2014). Unlike the analysis of the stomach food content, the fatty acid signature of mussels' tissues can reveal bivalves' food sources in a long-term basis (Ezgeta-Balić et al., 2012). Mussels are generalist filter-feeders that feed on seston and could potentially incorporate the fatty acids signatures of the diatoms, dinoflagellates, bacteria and other suspended particulate organic matter sources into their tissues. The fluctuations in the FA composition of the mussels' diet can influence their growth and lipid composition (Alkanani et al., 2007; Narváez et al., 2008). Freites et al. (2002a) found that the nutritional quality of the natural seston explained the variance of almost all FA of energetic importance to mussel *Mytilus galloprovincialis*, even if selective retention was observed for 20:4ω6 during winter for reproductive processes rather than food ingestion.

From a bottom-up point of view, certain FA and FA ratios can be used as biomarkers to characterize the seasonal contribution of phytoplankton classes and bacteria to the mussels' diet

(Budge et al., 2001; Handå et al., 2012a). Diatoms are rich sources of 16:1ω7 and 20:5ω3 (eicosapentaenoic acid or EPA) and are characterize by a ratio 16:1ω7 / 16:0 > 1 and 20:5ω3 / 22:6ω3 > 1 (Budge et al., 2001; Dalsgaard et al., 2003). On the other hand, FA 16:0, 18:1ω9, 18:4ω3, 22:6ω3 (docosahexaenoic acid or DHA) together with the ratio 22:6ω3 / 20:5ω3 > 1 are typical dinoflagellate markers (Budge et al., 2001; Dalsgaard et al., 2003). These distinct FA can be transferred from primary producers up to the mussel tissues and indicate a preferential ingestion of diatoms or dinoflagellates. In addition, odd-numbered branched FA like 15:0 and 17:0, 18:1ω7 and the ratio 18:1ω7 / 18:1ω9 > 1 can indicate a bacterial input in the mussels' diet (Budge et al., 2001).

Even if phytoplankton is the primary food source for mussels, several studies have reported that mussels can effectively utilize excess particulate organic matter coming from fish cages when reared in proximity to the net-pens (Handå et al., 2012a). The analysis of the fatty acid profile of mussels cultured near fish cages or directly exposed to fish effluents can be used to trace the assimilation of uneaten fish feed in the mussels' tissues (Gao et al., 2006; Redmond et al., 2010; George and Parrish, 2012; Both et al., 2011; 2012; 2013; Handå et al., 2012a). Monounsaturated FA (MUFA) 20:1ω9, 22:1ω11 and EPA are traditional fish feed biomarkers, originated from the sardine, herring and capelin utilized for feed manufacturing (NRC, 1993). Recently, fish feed have started to contain elevated amounts of vegetable fatty acids derived from terrestrial seed oils and meals that are not generally found in the marine food chains and thus, these terrestrial FA can be indicators of feed waste assimilation by mussels. These fish feed markers include high percentages of oleic acid 18:1ω9 and polyunsaturated fatty acids (PUFA) such as linoleic acid 18:2ω6, α-linolenic acid 18:3ω6 along with eicosenoic acid 20:1ω9, arachidonic acid (20:4ω6) and a low ω3/ω6 ratio (Gao et al., 2006; Redmond et al., 2010; Both et al., 2011; Handå et al., 2012a).

The principal objective of this study was to analyze the trophic interactions between seston and mussels *M. galloprovincialis* cultured in suspension in a commercial raft polygon in the Ría de

Ares-Betanzos (Galicia, N.W. Spain) using fatty acid trophic markers. Due to the fact that some of the mussel rafts are located proximate to a fish aquaculture site, a second aim of this study was to assess if mussels cultured in a raft near fish cages were assimilating any uneaten fish feed (i.e. from feed 'fines' up to particles <50 µm) as part of their diet. We compared the fatty acid profile of mussels cultured in a raft close to fish cages of red sea bream (*Pagellus bogaraveo*) with mussels reared in a raft distant from the net-pens. Insights on the effectiveness of the utilization of fish feed by mussels will be of great importance for the implementation of integrated mussel-fish systems worldwide, but especially for Galicia, a region where mussel farming is the principal aquaculture activity and provides 9,000 and 20,000 direct and indirect jobs, respectively (Labarta et al., 2004).

| Source | FA biomarkers | References |
|---|---|---|
| Fish feed | $18:1\omega9$ | Gao et al., 2006; Redmond et al., 2010; Handå et al., 2012a |
| | $18:2\omega6$ | Redmond et al., 2010; Both et al., 2011 |
| | $20:5\omega3$ (EPA) | NRC, 1993 |
| | $20:4\,\omega6$ | Both et al., 2011 |
| | $20:1\omega9$ | Both et al., 2012, 2013 |
| | $20:1\omega11$ | Both et al., 2012, 2013 |
| | $22:1\omega11$ | NRC, 1993; Both et al., 2012, 2013 |
| | low $\omega3/\omega6$ ratio | Redmond et al., 2010 |
| Diatoms | $16:1\omega7$ | Budge et al., 2001; |
| | $20:5\omega3$ (EPA) | |
| | $16:1\omega7/16:0 > 1$ | Dalsgaard et al., 2003 |
| | $20:5\omega3/ 22:6\omega3 > 1$ | |
| Dinoflagellates | $16:0$ | Budge et al., 2001; |
| | $18:1\omega9$ | |
| | $18:4\omega3$ | |
| | $22:6\omega3$ (DHA) | Dalsgaard et al., 2003 |
| | $22:6\omega3/ 20:5\omega3 >1$ | |
| Bacteria | $15:0$ | Budge et al., 2001 |
| | $17:0$ | |
| | $18:1\omega7$ | |
| | $18:1\omega7/ 18:1\omega9 > 1$ | |

Table 1. Summary of the principal fatty acid (FA) biomarkers used in this study to identify the food sources of mussels.

## 2. Material and methods

### 2.1. Study site

The Ría de Ares-Betanzos is located between Cape Fisterra and Cape Prior in Galicia, on the N.W. coast of the Iberian Peninsula (43°22'39.20''N, 8°12'39.77''W; Fig. 1). Seston and mussel samples were collected at two commercial mussel rafts in Lorbé polygon: raft P-14, placed in the proximity (170 m North) of fish net-pens (43°23'19.62''N, 8°17'17.71''W) and raft P-46, located further away (550 m North) from the cages (43°23'19.62''N, 8°17'17.71''W). Rafts P-14 and P-46 had the same dimensions (550 m$^2$) and mussels were cultured following the traditional commercial protocols (Fig. 1). Mussel seeds originated from the same location and the average density was 700 mussels m$^{-1}$ rope. Lorbé raft polygon is situated on the southern shore of the Ría Ares-Betanzos and is the main area of shellfish farming with 107 culture units grouped in parallel rows.

The Ría Ares-Betanzos covers an area of 52 km$^2$, a total volume of 0.65 km$^3$ and depths between 2 and 43 m (Sánchez-Mata et al., 1999; Álvarez-Salgado et al., 2011). The Ría is a double estuarine system (Asensio-Amor and Grajal-Blanco, 1981) that receives an average flow of 16.5 and 14.1 m$^3$ s$^{-1}$ from rivers Eume and Mandeo, respectively (Prego et al., 1999). The Ría Ares-Betanzos has great bio-economical importance owing in part to the extensive cultivation of mussel *M. galloprovincialis* on suspended rafts that produce 10,000 Tyr$^{-1}$ (Labarta et al., 2004). Mussel production per raft in the Galician Rías is estimated to range from 60 to 84 Tyr$^{-1}$ and the production cycle lasts 16-18 months, with production and seed ropes coexisting in the same raft to supply commercial demands (Labarta et al., 2004). Floating cages for the culture of red sea bream (*P. bogaraveo*) are installed within the inner part of Lorbé raft polygon. The fish farm has net-pens with a depth of 6 m and a rectangular base of 2.5 x 1.5 m (approximate volume of 3,692.64 m$^3$). A commercial diet of heat extruded fish pellets (Skretting B4 power 2 P) is supplied daily *ad limitum* and represents a ration of 0.5 to 0.7% of the fish weight (Guisado et al., 2007). The fish farm has an estimated annual production of 245 tonnes (JACUMAR, 2011).
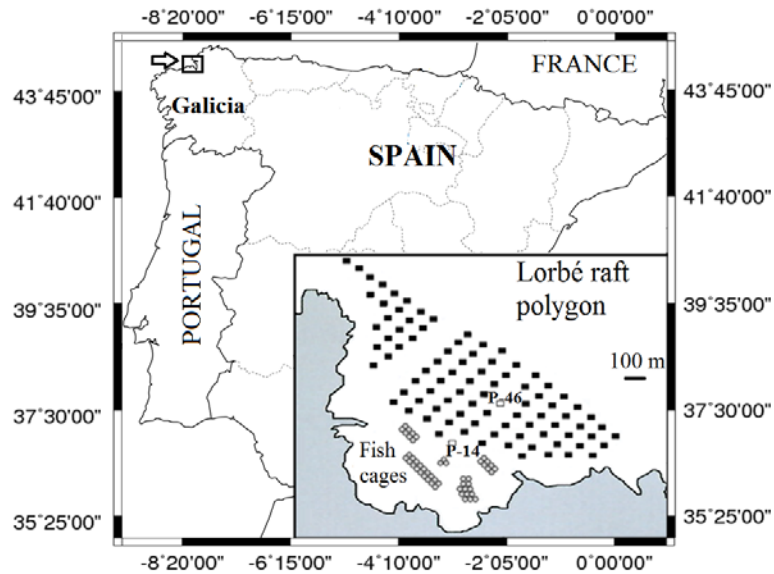
Fig. 1. Geographical position of Lorbé mussel raft polygon in the Ría Ares-Betanzos (Galicia, N.W. Spain). The white squares represent the mussel raft adjacent to the fish farm (P-14; 170 m from the cages) and the raft distant from the cages (P-46; 550 m from the cages). The black squares indicate the position of the remaining mussel rafts moored in the shellfish polygon.

## 2.2. Sampling strategy

The diet of the bivalves was analyzed by measuring the fatty acid profiles of the seston, mussels' organs (mantle and digestive gland) and feces collected during five different sampling events at both rafts, to evaluate seasonal and spatial variations in the feeding ecology. Mussel sampling was conducted on rafts P-14 and P-46 during two consecutive days on the months of July 2010 (summer), October 2010 (autumn), February 2011 (winter), May 2011 (spring) and October 2011 (autumn) in order to test for any seasonal variability. The two samples from October served as a test for inter-annual variance in the autumn. The sampling depth (2.0-3.0 m) was similar for the two rafts (P-14, P-46). Adult mussels (shell length: 50-70 mm) were

handpicked randomly from the suspended ropes and epibiotic organisms were removed from the shell before being dissected or placed in a mesocosm to collect the feces.

Mussels were dissected to obtain separate samples of the mantle tissue and the digestive gland. The organs of 3-5 individuals were pooled as one replicate. Three replicates were taken for each tissue (mantle and digestive gland) (n=15 mussels per site). Mussels were placed in a mesocosm of 19 l consisting of three replicate tanks, each divided into 16 compartments containing 3 to 4 mussels and one additional empty control tank (Filgueira et al., 2006). Seawater from 3 m depth was supplied by a peristaltic pump into a header tank at each raft and filtered through a 50 µm nylon mesh before being distributed at a constant flow rate of 3 l min$^{-1}$ into the mesocosm (Filgueira et al., 2006). Mussels were left undisturbed for 1 h after harvesting from the ropes. At the end of the mesocosm experiment, the deposited mussel feces from each tank were manually collected into a 25 ml glass vial by means of a pipette. Three replicates of mussel feces were collected per sampling site (raft), each replicate comprising the feces of 12 individuals (n=15 feces samples per site). Feces were filtered through ammonium formate (0.5 M) pre-washed and pre-combusted (450°C, 4 h) Whatman GF/F filters (0.7 µm nominal pore size) and rinsed with isotonic ammonium formate to remove salts. Filters were dried to constant weight. Three replicates of 5 l of seawater were collected from the empty control tank during each seasonal sampling trip to analyze the FA signature of the natural seston.

Seston samples were taken consecutively throughout the morning, coinciding with the time when red sea bream was manually fed from a fish barge and presumably, the maximum amount of uneaten feed particles was available in the water.

The water samples were filtered following the steps described for the feces. Three replicates of 350- 400 mg of the commercial fish pellets were collected directly from the feed barge in October 2011.

All samples were immediately frozen and stored at -50°C until lyophilization with a freeze-dryer (Ilshin Lab Co. Ltd, Korea). The dry samples were stored at -20°C until further homogenization with a mortar and a pestle or with an ultrasonic Branson Sonifier (250/450 USA) in the case of the mussel organs.

## 2.3. Fatty acids analyses

Total lipids were extracted according to the method of Bligh and Dyer (1959) modified by Fernández-Reiriz et al. (1989). As internal standard, 200 µl of nonadecanoic acid (19:0) diluted in pure hexane (100 µg acid / 1 ml hexane) was added to aliquots of 400 µg of total purified lipids. Samples were evaporated to dryness by flushing with nitrogen. Total lipids were quantified following the method described by Marsh and Weinstein (1966) with a tripalmitine standard (Sigma Aldrich Inc., Buchs, Switzerland). The fatty acids (FA) from the total lipids were transesterified to fatty acid methyl esters (FAME) with a solution of toluene and concentrated sulfuric acid solution in methanol (1.5:100 ml), according to Christie (1982). Air was flushed from the tubes, which were closed to avoid evaporation. Samples were kept at 50°C for 18-20 h. After cooling, 5 ml of 6% $K_2CO_3$ was added. The resultant emulsion was centrifuged at 3000 rpm for 5 min. The supernatant containing the total FAME was injected in a gas chromatographer (Perkin-Elmer, 8500) equipped with a flame ionization detector and a 30 m capillary column of flexible silica (Supelco, SP-2330). Nitrogen was used as the carrier gas at a pressure of 0.069 Pa. A programmable temperature injector with 275°C was used, operating in solvent elimination mode (Medina et al., 1994). The temperature of the column increased from 140°C to 210 °C at a rate of 1°C min$^{-1}$.

Total FAME obtained after transesterification of the mussel feces were purified by adsorption chromatography before being injected in the chromatograph. FAME samples were eluted with a mobile phase consisting of a mixture of hexane and ether (95:5 ml) following Christie (1982). Remains of polar impurities (i.e., cholesterol) were retained by the stationary phase of 99% silicic acid. The purified samples were collected and evaporated to dryness under a stream of nitrogen and resuspended with toluene.

Fatty acids were identified by co-injection of the samples along with standard mixtures of established composition in order to compare relative retention times.

Results for each fatty acid were expressed as the relative percentage (%) of the total fatty acid content ± standard deviation. Shorthand FA notations of the form A:BωX were used, where A

represents the number of carbon atoms, B gives the number of double bonds and X gives the position of the double bond closest to the terminal methyl group (Guckert et al., 1985). The FA markers used in this study are summarized in Table 1.

## 2.4. Statistical analyses

In this study we combined similarity-based multivariate analyses with univariate analyses (ANOVA) to examine: 1) differences in the overall FA composition between the two groups of samples (i.e. from rafts P-14 and P-46) and sampling time (i.e. seasons), and 2) transference of FA markers from food sources (seston and uneaten fish feed particles) to consumers (mussels' organs and feces). Multivariate analyses of FA compositions were conducted with a non-metric multidimensional scaling method (NMDS) based on Bray-Curtis similarity matrix using Primer 5 software (Clarke and Gorley, 2006). Relative FA concentrations (%) were logarithmically transformed prior to the NMDS analysis. The NMDS used the rank of similarities to provide a visual representation of any spatial and seasonal differences in the FA profile of the food source and the consumers. Stress values <0.05 indicated an excellent representation of the clusters and <0.1 and <0.2 indicated good and potentially useful plots, respectively. NMDS was performed in conjunction with two-way analysis of similarity (ANOSIM), to test whether clusters in the NMDS plot differed significantly from each other based on the sampling site and the season. The $R$ statistic of ANOSIM varies between -1 and 1: $R$ values close to 1 indicate well separated clusters and $R$ values close to 0 indicate a weak separation, this meaning a complete similarity of the samples. Lastly, similarity percentage analysis (SIMPER) was calculated with logarithmically transformed percentages to determine the main FA contributing to differences between samples. Differences in FA classes and specific FA biomarkers detected with the multivariate analyses were tested with a two-way analysis of variance (ANOVA) with Statistica 7 software (StatSoft 2004) with site and season as main independent variables. The assumptions of normality and homoscedasticity were checked with Shapiro-Wilk and Levene test prior to

ANOVA. Tukey HSD analysis was used for *post-hoc* pairwise comparisons using 95% confidence limits.

# 3. Results

## 3.1. Seston

The relative FA composition (%) of seston was dominated by the SAFA 16:0 and 18:0, the MUFA 16:1ω7 and 18:1ω9 and the PUFA 20:4ω6 (see supplementary material, Table S1). The FA classes consisted mainly of SAFA (average 46.3±12.6%), MUFA (29.4±7.5%) and PUFA in a lower proportion (12.3±3.4%) (Table S2). Diatom-specific FA markers were observed in the seston during all seasons, but with higher relative abundance in spring-summer than in autumn-winter. Diatom-specific fatty acid markers 16:1ω7 (9.01%) and the ratio EPA/DHA (1.67) were significantly higher in summer, whereas EPA (2.14%) dominated in spring (ANOVA, $p<0.05$). The ratio 16:1ω7/16:0 (0.39) showed the same value at both seasons. Diatom biomarkers showed significantly lower values in autumn and winter (16:1ω7 =7.31%, EPA=1.53%, 16:1ω7/16:0=0.25, EPA/DHA=1.20) (ANOVA, $p<0.05$) (Fig. 2 A). In general, seston showed a prevalence of dinoflagellate fatty acid markers in autumn and winter (16:0=27.72%, 18:1ω9=10.51%, 18:4ω3=1.20%, 22:6ω3=1.46%, DHA/EPA=1.12) compared to summer and spring (16:0=25.08%, 18:1ω9=9.30%, 18:4ω3=1.06%, 22:6ω3=1.50%, DHA/EPA=0.70) (Fig. 2 A). Specifically, significantly higher levels of 18:1ω9 and 22:6ω3 were found in autumn 2010 and 2011, respectively, and greater proportions of 16:0 and 18:4ω3 were computed in winter (ANOVA, $p<0.05$). Bacteria biomarkers were detected during all seasons, but with higher relative contribution in summer and spring (15:0=2.85%, 17:0=1.50%, 18:1ω7=6.50%, 18:1ω7/18:1ω9=1.20) than autumn and winter (15:0=3.30%, 17:0=1.78%, 18:1ω7=4.80%, 18:1ω7/18:1ω9=0.52), except for 17:0, that was significantly higher in the seston in winter (ANOVA, $p<0.05$) (Fig. 2A).

The two-way ANOSIM test and the NMDS plot indicated that there was a significant difference in the seston FA composition among seasons (Fig. 3A, stress 0.15, global *R*: -0.035, $p=0.05$),

although the spatial segregation was small and the highest dispersal of points was between the summer and autumn 2010 samples from the rest of the seasons. This spatial segregation suggested inter-annual differences in the FA composition of the seston during autumn 2010 and 2011. Temporal dissimilarity between seasons ranged between 12 and 15% and was mainly explained by diatom markers 16:1ω7 and 20:5ω3, that were especially abundant in summer and spring, respectively, and to the dinoflagellate marker 22:6ω3, that peaked in autumn 2011 (SIMPER, Fig. 4A-C). The similarity of the replicate samples within each season were 87.2 and 87.8% for autumn and spring, and 92.3 and 92.4% for summer and winter, respectively (SIMPER). Similarity within seasons was mainly attributed to FA 16:0, 16:1ω7 and 18:0, some of the most abundant FA contained in the seston (Table 2). On the other hand, the NMDS revealed a weak spatial separation regarding the sampling location (Fig. 3B, stress 0.15, two-way ANOSIM, global $R$: 0.308, $p=0.006$), because dissimilarity between sampling sites was low (average dissimilarity 10%), and attributed to FA 20:4ω6, 18:1ω9 and 20:1ω9 (SIMPER, 9.9, 8.1 and 8.0%, respectively). Two-way ANOVA revealed no significant spatial effect for the fish feed markers 20:4ω6 ($F_{(4, 20)}=0.90$, $p=0.34$), 18:1ω9 ($F_{(4, 20)}=0.62$, $p=0.44$) or 20:1ω9 ($F_{(4, 20)}=0.86$, $p=0.37$), even if the former were the main FA contributing to dissimilarity among rafts. The average similarity within the raft distant and close to the fish cages was 86.7% and 87.3%, respectively. These high similarities were attributed to 14:0, 16:0 and 16:1ω7.

Seasonality of some FA classes was evident (ANOVA, Table 3). SAFA was significantly higher in spring than in summer (Tukey HSD, $p<0.001$). Although ANOVA showed no seasonal differences in the overall PUFA content ($p>0.05$), PUFA ω3 were significantly higher in spring, winter and autumn 2011 than the rest of the seasons ($F_{(4, 20)}=6.11$, $p<0.001$). The ratio ω3/ω6 followed the same pattern. The concentration of NMI was higher in autumn 2011 than in the other seasons ($p<0.001$). Two-way ANOVA showed a spatial and temporal effect on the content of NMID (Table 3), with higher NMID concentrations in the seston sampled in autumn 2011 close to the fish cages in comparison to the raft further away.
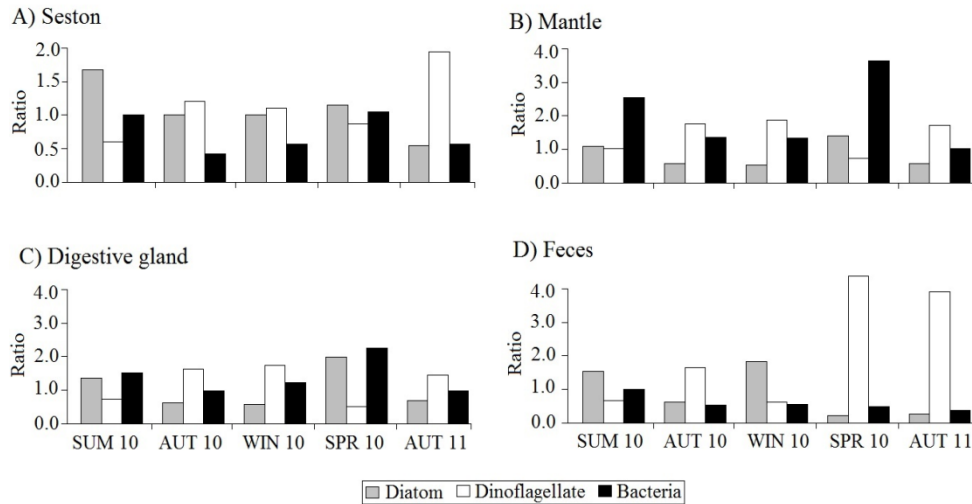
Fig. 2. Seasonal variations of the principal fatty acid ratios typical of diatoms (20:5ω3/22:6ω3>1), dinoflagellates (22:6ω3/20:5ω3>1) and bacteria (18:1ω7/18:1ω9>1) measured for: A) the seston, B) mantle, C) digestive gland and, D) feces of mussel *M. galloprovincialis*.

## 3.2. Mussel: mantle tissue

The most important FA in the mantle tissue were SAFA palmitic acid 16:0, MUFA 16:1ω7, while among PUFA, EPA (20:5ω3) and DHA (22:6ω3) were the most abundant (Table S3). In terms of relative composition, PUFA (53.7±2.9%) represented the highest percentage, followed by SAFA (25.9±1.8%) and MUFA (13.7±2.3%) (Table S4).

Diatom biomarkers dominated the mantle composition in summer and spring (16:1ω7=7.31%, EPA=19.50%, 16:1ω7/16:0=0.40, EPA/DHA=1.40) and diminished during autumn and winter (16:1ω7= 4.44%, EPA=14.89%, 16:1ω7/16:0=0.20, EPA/DHA= 0.56). A shift toward higher levels of dinoflagellates biomarkers was found in autumn 2010 and 2011 and winter (16:0=19.21%, 18:1ω9=1.51%, 18:4ω3=1.88%, 22:6ω3=26.55%, DHA/EPA=1.80) in comparison with summer and spring (16:0=17.85%, 18:1ω9=0.97%, 18:4ω3=1.90%, 22:6ω3=16.75%, DHA/EPA=0.80) (Fig. 2 B). Bacteria FA markers predominated in the mantle during summer and spring (15:0=0.47%, 17:0=0.67%, 18:1ω7=2.90%, 18:1ω7/18:1ω9=3.09)

and were least abundant in the autumn-winter seasons (15:0=0.48%, 17:0=0.74%, 18:1ω7=1.80%, 18:1ω7/18:1ω9=1.24), although 17:0 was significantly higher in the mantle in winter (ANOVA, $p<0.05$) (Fig. 2B).

The NMDS confirmed that the FA composition of the mantle in spring and summer was clearly different to that during autumn and winter (Fig. 3 C, stress 0.06, two-way ANOSIM, global $R$: 0.674, $p=0.001$). Seasonal dissimilarity varied from 10 to 20% and was pointed by diatom biomarkers 16:1ω7 and 20:5ω3, that showed higher values in spring and summer, and to the dinoflagellate marker 22:6ω3, that peaked in autumn and winter as indicated above (Fig. 4D-F). The similarity within each season was very high (SIMPER, average similarity: 94%) and was mainly attributed to 16:0, EPA and DHA, the predominant FA of the mantle (Table 2).

| | Seston | | | Mantle | | | Digestive gland | | | Feces | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 16:0 | 16:1ω7 | 18:0 | 16:0 | 20:5ω3 | 22:6ω3 | 16:0 | 20:5ω3 | 22:6ω3 | 14:0 | 16:0 | 16:1ω7 |
| Summer | 12.21 % | 8.58 % | 8.43 % | 19.33 % | 17.65 % | 17.33 % | 10.09 % | 9.43 % | 8.42 % | 6.97 % | 12.10 % | 8.34 % |
| Autumn | 13.59 % | 8.17 % | < 5 % | 20.96 % | 15.36 % | 26.03 % | 8.30 % | 8.54 % | 9.95 % | < 5 % | 13.22 % | 7.52 % |
| Winter | 12.66 % | < 5 % | 8.13 % | 18.80 % | 16.15 % | 29.44 % | 9.10 % | 8.45 % | 10.13 % | 7.07 % | 12.87 % | 8.35 % |
| Spring | 13.81 % | 9.26 % | 9.42 % | 18.27 % | 23.27 % | 15.16 % | 9.32 % | 9.57 % | 7.35 % | 7.24 % | 12.33 % | < 5 % |

Table 2. SIMPER similarity percentages of most common fatty acid (FA) contributions within seasons obtained for seston, mussels' organs and feces.

The NMDS reveal a weak spatial separation in the mantle FA profile as samples from both sites were mixed (Fig. 3D, stress 0.06, ANOSIM, global $R$: 0.196, $p=0.051$), since spatial dissimilarity was low (average 9%), being 16:1ω7 and 14:0 the main contributors to dissimilarity between rafts (SIMPER, 8.4% and 7.3%, respectively). Average similarity within the raft distant and the one close to the fish cages was 90.1% and 91.6%, respectively, and was explained by 16:0, 20:5ω3 and 22:6ω3 for both sites.

Two-way ANOVA showed a temporal effect on most FA classes (Table 3). SAFA and MUFA were significantly higher in the mantle in summer than in winter and autumn (Tukey HSD, $p<0.001$). PUFA, DMA 18:0 and the ratio ω3/ω6 were higher in autumn and winter than in the

summer and spring (Tukey HSD, $p<0.001$). NMIT concentrations were higher in autumn and winter compared to spring (Tukey HSD, $p<0.001$).
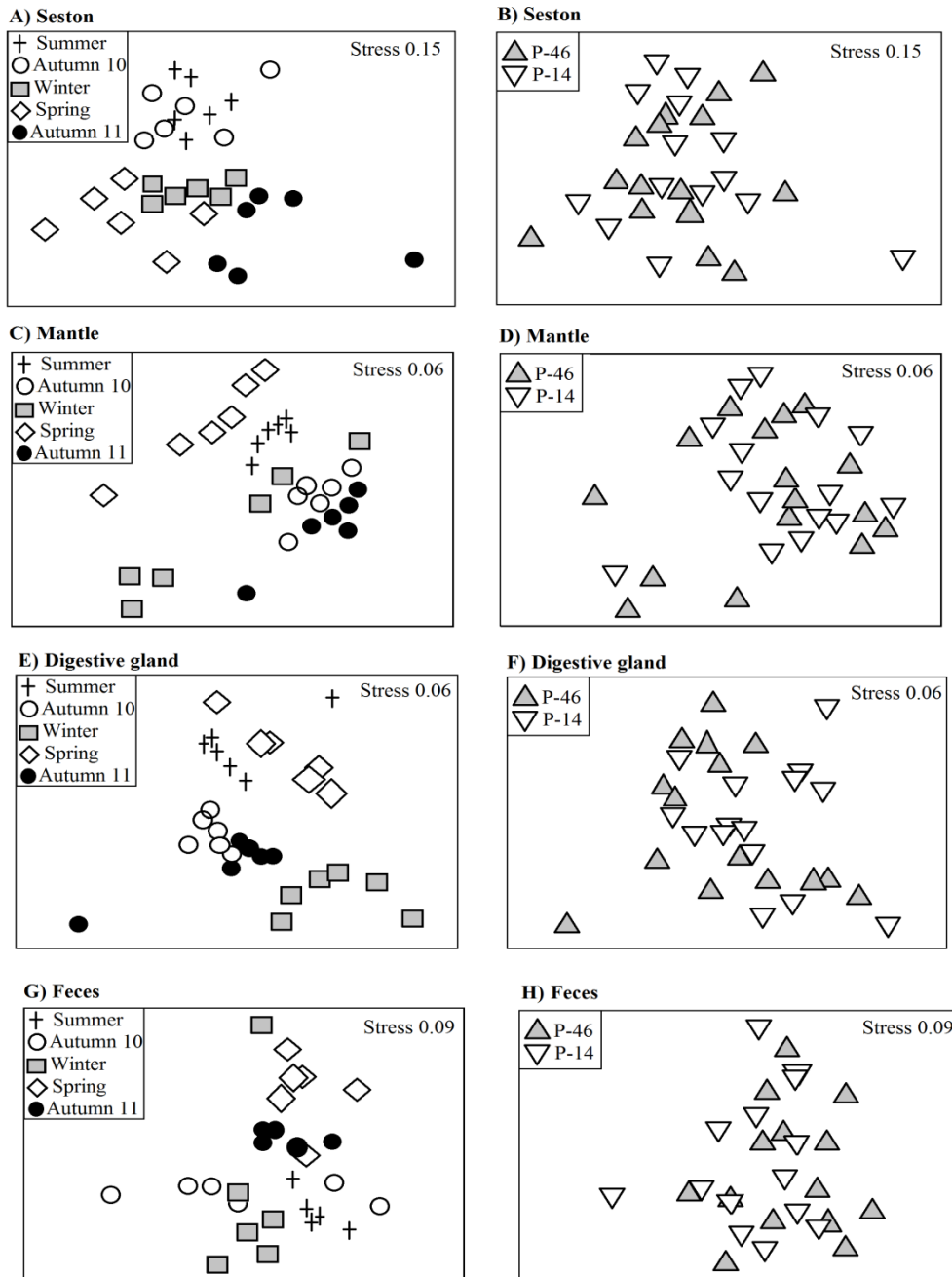


Fig.3. Non-metric multidimensional scaling (Bray-Curtis similarity) on log transformed relative FA content (%) for (A-B) the seston, (C-D) mantle, (E-F) digestive gland and (G-H) feces sampled during five different seasons (left panels) and at two rafts (right panels) distant (P-46) and close (P-14) from fish cages.

### 3.3. Mussel: digestive gland

The FA profile of the digestive gland was very similar to the one reported for the mantle (Tables S5 and S6). Likewise, the gland was a diatom-dominated organ in spring (EPA/DHA=2) and summer (EPA/DHA=1.35), whereas dinoflagellates biomarkers were most important in autumn 2010 and 2011 and winter (DHA/EPA=1.63, 1.44 and 1.74, respectively). The digestive gland contained higher proportions of bacterial biomarkers during spring ($18:1\omega7/18:1\omega9 = 2.25$) and summer ($18:1\omega7/18:1\omega9 = 1.52$) than in the autumn-winter ($18:1\omega7/18:1\omega9 = 1.0$) (Fig. 2C).

The NMDS plot showed a strong temporal separation between winter, spring and summer and autumn (i.e. warmer *vs* colder seasons) (Fig. 3E, stress 0.06, two-way ANOSIM, global *R*: 0.801, $p=0.001$). Temporal dissimilarity was driven by the same FA as the mantle and varied from 8 to 16% (Fig. 4G-I). The similarity within each season was very high (SIMPER, average similarity: 94%) and was mainly attributed to 16:0, EPA and DHA (Table 2).

The NMDS didn't reveal any strong spatial separation regarding the sampling location (Fig. 2F, stress 0.06, two-way ANOSIM, global *R*: 0.196, $p=0.054$). FA $16:1\omega7$ and DMA 18:0 were the main contributors to dissimilarity between rafts (SIMPER, 8.4% and 7.8%, respectively). Average similarity within both sampling sites was high, with 89.0% for P-46 and 91.6% for P-14. Similarity within rafts was explained by the same FA as for the mantle samples.

Two-way ANOVA showed a significant effect of the site, season and the interaction term (site x season) on most FA classes (Table 3). SAFA and MUFA were significantly higher in the digestive gland in summer and spring, while PUFA displayed an opposite pattern (Table 3, Tukey HSD, $p<0.001$). DMA 18:0 was higher in the raft closer to the fish cages than in the site distant to the cages (Tukey HSD, $p<0.001$). In addition, levels of DMA 18:0 were higher in winter than in the other seasons (Tukey HSD, $p<0.001$) (Table 3). The ratio $\omega3/\omega6$ was higher in spring and autumn 2011 than in the other seasons (Tukey HSD, $p<0.001$). NMI, NMIT and NMID were present in higher concentrations in the gland of mussels collected close to the fish cages during winter, autumn 2010 and spring in comparison to the ones collected further away from the fish cages (P-46) in the same seasons (Tukey HSD, $p<0.001$).
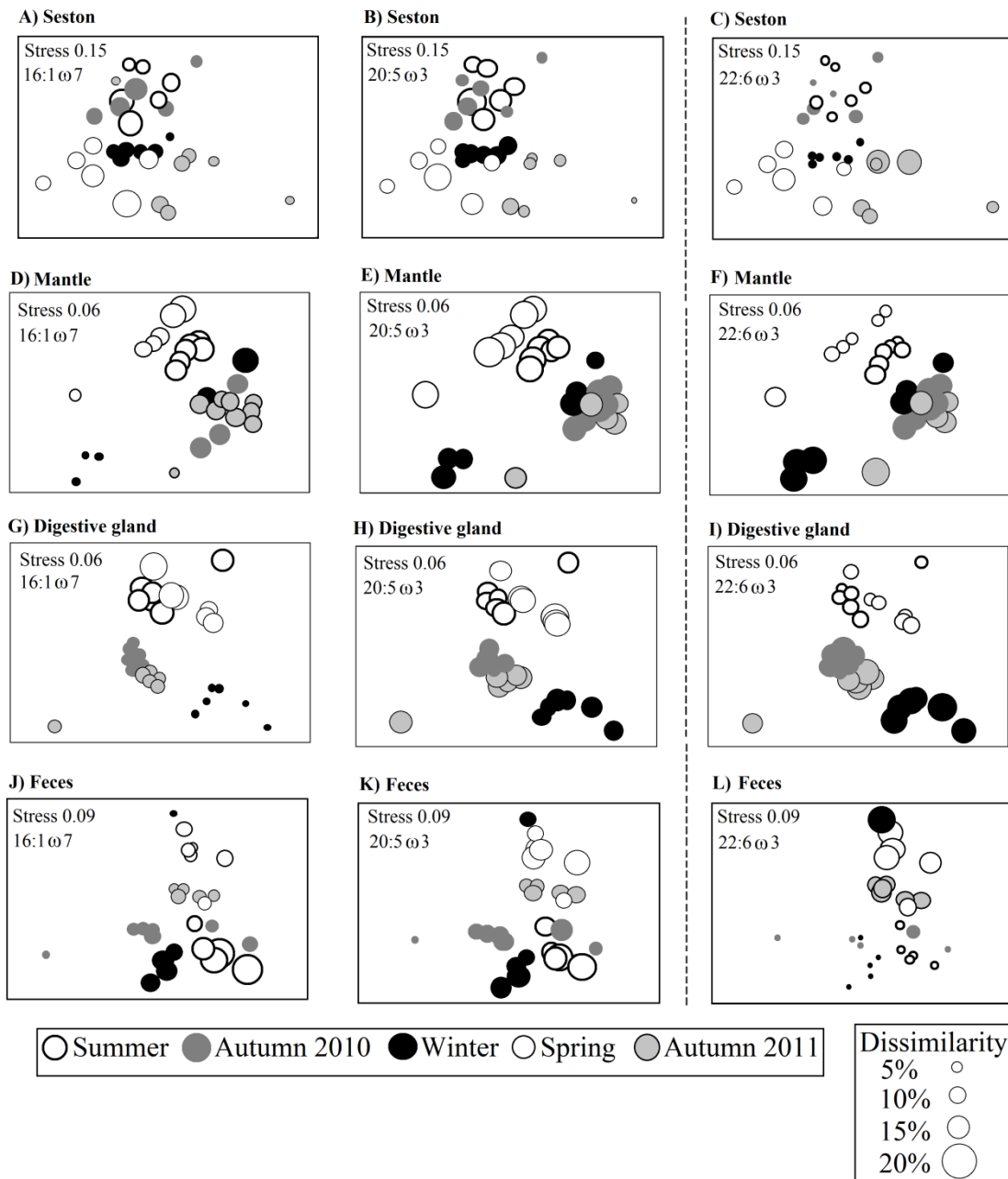
Fig. 4. Bubble plots of the FA that explained the highest dissimilarity between seasons (SIMPER) for the seston (A, B, C), mantle (D, E, F), digestive gland (G, H, I) and mussel feces (J, K, L). Plots are arranged with the diatom-indicating FA biomarkers on the left (16:1ω7 and 20:5ω3) and dinoflagellate biomarkers on the right (22:6ω3).

## 3.4. Mussel: feces

The FA profile of feces of mussels consisted mainly of 16:0, 14:0 and 16:1ω7 (Table S7). SAFA (51.5 ±4.9%) was the most abundant class, followed by PUFA (25.0±6.8%) and MUFA (21.5±6.0%) (Table S8). The feces contained significantly higher amounts of diatom biomarkers 16:1ω7 (14.36%) and EPA (5.73%) during the summer, as well as higher diatom-specific ratios 16:1ω7/16:0=0.35 and EPA/DHA=1.54 (ANOVA, $p<0.05$) (Fig. 2D). EPA was also abundant in spring (4.67%), whereas high levels of 16:1ω7 (10.35%) and EPA/DHA=1.82 were recorded in winter. Biodeposits sampled in autumn and winter showed significantly higher levels of dinoflagellate biomarkers 18:1ω9 (6.00%) (ANOVA, $p<0.05$) and a relative high content of 16:0 (28.42%), 18:4ω3 (2.33%), 22:6ω3 (7.00%) and DHA/EPA=3.40 (Fig. 2D). Significantly higher levels of DHA (20.38%), 18:4ω3 (6.30%) and an elevated DHA/EPA ratio of 4.37 were recorded in the feces during spring (ANOVA, $p<0.05$). Bacteria biomarkers were most abundant during summer (15:0=1.56%, 17:0=2.00%, 18:1ω7=4.04%, 18:1ω7/ 18:1ω9=1.1), although 15:0 (2.63%) and 17:0 (2.76%) peaked during the winter and autumn 2010, respectively (ANOVA, $p<0.05$).

The NMDS plot showed a significantly different seasonal trend (Fig. 3G, stress 0.09, two-way ANOSIM, global $R$: 0.45, $p=0.001$). Temporal dissimilarity ranged from 16 to 21% and was attributed to the diatom biomarkers 16:1ω7 and 20:5ω3, as well as to dinoflagellate biomarker 22:6ω3 (Fig. 4 J-L). The similarity within each season was high (SIMPER, average similarity: 85%) and pointed out by FA 16:0, 16:1ω7 and 14:0 (Table 2).

There was no significant spatial separation for the feces samples (Fig. 3H, stress 0.09, two-way ANOSIM, global $R$: -0.013, $p=0.053$), due to a low dissimilarity between rafts (10.8%) explained by FA 22:0, 22:6ω3 and 18:0 (SIMPER, 10.7, 8.2 and 7.8%, respectively). Average similarity within raft P-46 and P-14 was 82.9 and 82.6%, respectively. FA responsible for similarity within rafts were 16:0, 16:1ω7 and 14:0.

Two-way ANOVA showed a significant effect of the site, season and the interaction term (site x season) on many FA classes (Table 3). Feces contained significantly higher amount of SAFA in

mussels sampled further away from the fish cages in winter compared to the other raft (Tukey HSD, $p<0.001$). MUFA and PUFA were higher in spring and autumn 2011 than the other seasons ($p<0.001$). DMA 18:0 was higher in the raft distant from the fish cages in summer and autumn 2010, while the ratio ω3/ω6 of the feces was higher in spring ($p<0.001$). NMI, NMID and NMIT were higher in the raft distant from the cages during autumn 2010 than in the raft adjacent to the fish in the same season ($p<0.001$).

### 3.5. Comparisons with fish feed

The relative FA composition (%) of fish feed comprised high proportions of 16:0, 18:1ω9, EPA and DHA. PUFA was the most common class (41.0%), followed by MUFA (30.7%) and SAFA (27.4%) in similar quantities (Table S9). An overall NMDS plot including all samples showed that the relative FA composition of seston and mussel samples largely differed from that of the fish feed (Fig. 5, stress 0.08, two-way ANOSIM, global $R$: 0.76, $p<0.01$). Dissimilarity between seston and fish feed, both representing potential food for the mussels, was mainly explained by 16:0 (10.3%), 16:1ω9 (10.0%) and 18:0 (9.1%) (SIMPER, average dissimilarity 51.2%). The FA composition of the mantle and the digestive gland grouped together but diverged clearly from the potential food sources (fish feed, seston) and the feces. The dissimilarity between the fish feed and the mantle was mainly explained by 22:6ω3 (9.6%), DMA 18:0 (8.6%) and 20:5ω3 (7.4%) (SIMPER, 53.4%); while the seston and the mantle mainly differed in the content of 22:6ω3 (9.8%), 20:5ω3 (8.2%) and 16:1ω9 (7.9%) (SIMPER, 39.1%). On the other hand, average dissimilarity between the feed and digestive gland was mainly explained by 22:6ω3 (7.8%), 20:5ω3 (7.4%) and 16:0 (7.2%) (SIMPER, 53.4%); while seston and the gland mainly differed in terms of 22:6ω3 (9.0%), 20:5ω3 (8.1%) and 16:1ω9 (7.8%) (SIMPER, 35.7%). The mantle and digestive gland showed a similar profile (SIMPER, 11.9%) and dissimilarity was attributed to 14:0 (8.6%), 16:1ω7 (8.5%) and DMA 18:0 (6.2%). Lastly, the overall NMDS plot showed a strong separation between the feed and the feces (SIMPER, 50.6%), mainly explained by 16:0 (10.2%), 22:0 (7.2%) and 18:0 (7.2%). Separation between

the seston and the feces was considerably smaller than for seston-feed (SIMPER, average dissimilarity 23.3%) and was attributed to 22:6ω3 (8.9%), 22:0 (7.3%) and 16:1ω9 (6.2%). Dissimilarity between the feces and the mantle was best explained by 22:0 (8.7%), 20:5ω3 (7.5%) and 22:6ω3 (6.7%) (SIMPER, 27.0%); while the feces and gland mainly differed in 22:0 (8.5%), 20:5ω3 (7.4%) and 16:1ω9 (6.2%) (SIMPER, 24.2%).
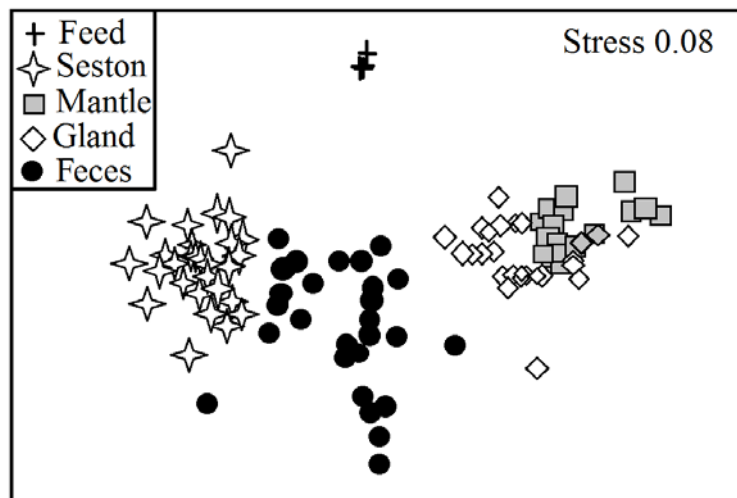


Fig. 5. Non-metric multidimensional scaling (Bray-Curtis similarity) on log transformed relative FA content (%) for fish feed, seston, mantle, digestive gland and feces sampled during five seasons at the rafts distant and close from the fish cages.

| | Site | | | | Season | | | | Site x season | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SS | MS | $F$-value (4,20) | $p$-value | SS | MS | $F$-value (4,20) | $p$-value | SS | MS | $F$-value (4,20) | $p$-value |
| **Seston** | | | | | | | | | | | | |
| SAFA | 5.42 | 5.42 | 0.09 | 0.76 ns | 804.09 | 201.02 | 3.30 | < 0.05 * | 63.81 | 15.95 | 0.26 | 0.89 ns |
| MUFA | 42.04 | 42.04 | 0.88 | 0.35 ns | 240.38 | 60.09 | 1.26 | 0.31 ns | 224.38 | 56.1 | 1.18 | 0.34 ns |
| PUFA | 0.03 | 0.03 | 0 | 0.98 ns | 725 | 181.25 | 2.58 | < 0.05 * | 119.13 | 29.78 | 0.42 | 0.78 ns |
| DMA | 0.01 | 0.01 | 0.24 | 0.62 ns | 0.33 | 0.08 | 1.37 | 0.27 ns | 0.42 | 0.11 | 1.76 | 0.17 ns |
| ω3/ ω6 | 5.63 | 5.63 | 0.13 | 0.71 ns | 1205.7 | 301.42 | 7.09 | < 0.001 *** | 186.2 | 46.55 | 1.1 | 0.38 ns |
| NMI | 16.13 | 16.13 | 0.89 | 0.36 ns | 1126.3 | 281.58 | 15.59 | < 0.001 *** | 78.87 | 19.72 | 1.09 | 0.39 ns |
| NMID | 12.03 | 12.03 | 2.19 | 0.15 ns | 1152 | 288 | 52.44 | < 0.001 *** | 88.13 | 22.03 | 4.01 | < 0.05 * |
| **Mantle** | | | | | | | | | | | | |
| SAFA | 0.64 | 0.64 | 0.73 | 0.43 ns | 73.7 | 18.43 | 21 | < 0.001 *** | 8.87 | 2.22 | 2.53 | 0.07 ns |
| MUFA | 9.96 | 9.96 | 2.3 | 0.14 ns | 85.9 | 21.47 | 4.97 | < 0.001 *** | 33.57 | 8.39 | 1.94 | 0.14 ns |
| PUFA | 2.25 | 2.25 | 0.64 | 0.43 ns | 158.09 | 39.52 | 11.3 | < 0.001 *** | 53.54 | 13.38 | 3.83 | 0.01 ns |
| DMA | 0.74 | 0.74 | 0.84 | 0.37 ns | 23.56 | 5.89 | 6.7 | < 0.001 *** | 2.82 | 0.7 | 0.8 | 0.53 ns |
| ω3/ ω6 | 0.92 | 0.92 | 3.57 | 0.07 ns | 15.55 | 3.89 | 15.12 | < 0.001 *** | 2.66 | 0.67 | 2.59 | 0.07 ns |
| NMI | 3.54 | 3.54 | 3.93 | 0.06 ns | 9.27 | 2.32 | 2.57 | 0.07 ns | 0.81 | 0.2 | 0.23 | 0.92 ns |
| NMID | 2.94 | 2.94 | 4.11 | 0.05 ns | 9.14 | 2.28 | 3.19 | 0.03 ns | 0.78 | 0.2 | 0.27 | 0.89 ns |
| NMIT | 0.03 | 0.03 | 2.13 | 0.15 ns | 0.37 | 0.09 | 7.09 | < 0.001 *** | 0.02 | 0.01 | 0.39 | 0.81 ns |
| **Digestive gland** | | | | | | | | | | | | |
| SAFA | 132.3 | 132.3 | 4.2 | 0.06 ns | 1366 | 341.5 | 10.83 | < 0.001 *** | 118.53 | 29.63 | 0.94 | 0.46 ns |
| MUFA | 37.78 | 37.78 | 8.41 | < 0.01 ** | 544.94 | 136.24 | 30.32 | < 0.001 *** | 48.56 | 12.14 | 2.7 | 0.05 ns |
| PUFA | 22.2 | 22.2 | 2.44 | 0.13 ns | 1181.2 | 295.29 | 32.51 | < 0.001 *** | 23.27 | 5.82 | 0.64 | 0.63 ns |
| DMA | 288.3 | 288.3 | 10.22 | < 0.001 *** | 1142.7 | 285.67 | 10.13 | < 0.001 *** | 252.53 | 63.13 | 2.24 | 0.10 ns |
| ω3/ ω6 | 0.4 | 0.4 | 4.04 | 0.06 ns | 5.38 | 1.34 | 13.71 | < 0.001 *** | 1.09 | 0.27 | 2.79 | 0.06 ns |
| NMI | 4.16 | 4.16 | 29.59 | < 0.001 *** | 53.58 | 13.4 | 95.25 | < 0.001 *** | 7.22 | 1.81 | 12.84 | < 0.001 *** |
| NMID | 0.09 | 0.09 | 14.86 | < 0.001 *** | 2.99 | 0.74 | 111.57 | < 0.001 *** | 0.11 | 0.02 | 4.36 | < 0.001 *** |
| NMIT | 0.09 | 0.09 | 14.86 | < 0.001 *** | 2.99 | 0.74 | 111.57 | < 0.001 *** | 0.11 | 0.02 | 4.36 | < 0.001 *** |
| **Feces** | | | | | | | | | | | | |
| SAFA | 12.03 | 12.03 | 0.35 | 0.56 ns | 469.67 | 117.42 | 3.38 | < 0.05 * | 1071.80 | 267.95 | 7.72 | < 0.001 *** |
| MUFA | 2.29 | 2.29 | 0.50 | 0.48 ns | 879.25 | 219.81 | 47.75 | < 0.001 *** | 111.22 | 27.81 | 6.04 | < 0.001 *** |
| PUFA | 2.16 | 2.16 | 0.17 | 0.68 ns | 1163.24 | 290.81 | 23.40 | < 0.001 *** | 71.71 | 17.93 | 1.44 | 0.25 ns |
| DMA | 3.52 | 3.52 | 21.15 | < 0.001 *** | 5.89 | 1.47 | 8.85 | < 0.001 *** | 4.63 | 1.16 | 6.96 | < 0.001 *** |
| ω3/ ω6 | 5.86 | 5.86 | 2.46 | 0.13 ns | 785.29 | 196.32 | 82.39 | < 0.001 *** | 6.22 | 1.55 | 0.65 | 0.63 ns |
| NMI | 17.40 | 17.40 | 42.23 | < 0.001 *** | 18.47 | 4.62 | 11.21 | < 0.001 *** | 10.68 | 2.67 | 6.48 | < 0.001 *** |
| NMID | 13.86 | 13.86 | 36.03 | < 0.001 *** | 14.01 | 3.50 | 9.11 | < 0.001 *** | 7.52 | 1.88 | 4.89 | < 0.01 ** |
| NMIT | 0.20 | 0.20 | 39.19 | < 0.001 *** | 0.55 | 0.14 | 26.63 | < 0.001 *** | 0.30 | 0.08 | 14.73 | < 0.01 ** |

Table 3. Sum and mean squares (SS, MS), $F$-ratio and $p$-values results from two-way ANOVA for the effects of site and season on principal FA classes in seston, mantle, digestive gland and feces. Significance levels are denoted as $p<0.05$*, $p<0.01$**, $p<0.001$***, ns (not significant). SAFA = saturated FA, MUFA = monounsaturated FA, PUFA = polyunsaturated FA, DMA= dimethyl acetals FA, PUFA ratio for the ω3 and ω6 series, NMI= non-methylene-interrupted FA, NMID= NMI dienoic FA, NMIT= NMI trienoic FA.

## 4. Discussion

This study confirmed that fatty acid (FA) biomarkers are a powerful proxy to study temporal variations and trophic interactions between filter-feeders and seston (Budge et al., 2001; Wong et al., 2008; Prato et al., 2010; Ezgeta-Balić et al., 2012). Our data corroborated several studies that observed how the seasonal shifts in seston composition (Tiselius et al., 2012) and phytoplankton communities (Budge et al., 2001; Parrish et al., 2005) are reflected in changes in the proportions of their fatty acid biomarkers. Seston typically constitutes a mixture of phytoplankton, bacteria, protozoa, detritus and mineral particles (Newell, 1965; Bayne and Widdows, 1978). The experimental design of this study reflected marked changes in seston composition that occur during the four most representative oceanographic events of the Galician Rías: i) summer upwelling, ii) spring and autumn bloom, and iii) winter mixing (Figueiras et al., 2002; Alvárez-Salgado et al., 2008). Local upwelling events during spring and summer (March-September) enhance the positive residual circulation pattern of the Rías, resulting in the transportation of dinoflagellates to the continental shelf, leaving the embayment dominated by diatoms (Figueiras et al., 2002; Álvarez-Salgado et al., 2008). On the other hand, the reversal of the circulation pattern during downwelling (October-February) promotes a mixture of diatoms and dinoflagellates (Figueiras et al., 2002). SIMPER results confirmed this pattern, as the diatom biomarkers 16:1ω7 and 20:5ω3 and the ratio 20:5ω3/22:6ω3>1 were present during the entire year, but with larger relative abundance during spring and summer. The spatial separation between the seston samples in autumn 2010 and 2011 pointed out by the NMDS plot highlighted the enhanced contribution of the dinoflagellate marker 18:1ω9 in autumn 2010 and 22:6ω3 in autumn 2011. Inter-annual differences could be explained by the duration of thermal stratification events in the summer, as a consequence of varying annual intensities of the upwelling events (Figueiras et al., 2002; Alvárez-Salgado et al., 2008; Villegas-Ríos et al., 2011). Concurrently, the chlorophyll-*a* content was found to be significantly higher in autumn 2011 than in 2010 in the same raft stations during a parallel study (Irisarri et al., 2014).

The seasonal changes in the FA signature of the seston showed to govern the temporal fluctuations in the FA signature of the consumer *M. galloprovincialis*. The phytoplankton contained in the seston has been demonstrated to be the main diet of mussels (Handå et al., 2012a; Richoux et al., 2014). Mussels and phytoplankton have a mutual trophic relationship in which the former exploit the microalgae as a food source and the latter use the organic and inorganic metabolic wastes of the bivalves as nutrients (Newell, 2004). Mussels' organs reflected the fluctuations in the seasonal cycle of phytoplankton productivity typical for the Galician Rías. The high concentration of 16:1ω7, EPA and the ratio EPA/DHA>1 in the digestive gland and mantle tissue in spring and summer confirmed the prevalence of diatoms as a food input during the warmer upwelling seasons. On the other hand, the higher content of DHA, 18:1ω9, 18:4ω3 and DHA/EPA>1 in both organs in autumn and winter showed that dinoflagellates dominated the diet during the colder downwelling seasons (Table 1). These results agreed with Freites et al. (2002a), who found higher levels of 16:1ω7 and EPA in the bulk tissue of *M. galloprovincialis* from a Galician Ría during spring, whereas mussels were rich in DHA in winter. Similarly, Handå et al. (2012a) detected a higher proportion of diatom markers in the mantle and digestive gland of *M. edulis* during spring, while a shift toward a dinoflagellate-dominated diet was detected in late summer (August). In addition, FA distinctive of bacteria were found in high concentrations in the seston in May and July, so it is possible that mussels included them as a dietary intake, since we found high amounts of 18:1ω7 and a ratio 18:1ω7/18:1ω9>1 in the organs during the upwelling seasons. Bacteria have been previously described to colonize suspended phytoplankton cells and to be ingested by filter-feeding bivalves (Xu and Yang, 2007).

In the present study, however, temporal differences in seston quantity did not seem to be associated with differences observed in the bivalves' FA signature. Firstly, phytoplankton blooms have been reported to exceed 2400 mg C m$^{-2}$ d$^{-1}$ in the Ría Ares-Betanzos, while primary productivity in winter is scarce, with values lower than 20 mg C m$^{-2}$ d$^{-1}$ (Bode and Varela, 1998). Secondly, our data analysis was performed on a relative percentage of FA and

not in absolute terms. Therefore, seasonal variations in seston quality (composition) were the main factor reflected in mussels' FA composition.

Previous studies have mostly focused on the FA composition of the bulk tissue of *M. galloprovincialis* (Fernández-Reiriz et al., 1996; Freites et al., 2002 a;b; Xu and Yang, 2007; Prato et al., 2010), and information on the FA composition of the digestive gland and the mantle is scarce. In our study, the predominant FA found in the mantle were 16:0 (18.6%), 16:1ω7 (5.5%), EPA (20:5ω3) and DHA (22:6ω3), with 16.7 and 22.6%, respectively. These were also the major fatty acids found in the mantle of the green mussel *Perna viridis* collected from the field by Shin et al. (2008). The digestive gland of the mussels sampled in our study was also characterized by high levels of 16:0 (17.6%), 16:1ω7 (9.9%), EPA (14.8%) and DHA (16.9%). Ezgeta-Balić et al. (2012) reported that 16:0 (14.0%), 16:1ω7 (3.8%), EPA (22.3%) and DHA (26.41%) were the predominant fatty acids found in the digestive gland of *M. galloprovincialis*. In the present study, the digestive gland seemed to present the strongest response to the seasonal fluctuations in the seston' FA composition in comparison with the mantle. The digestive gland is a food processing organ with a fast turnover rate and has been demonstrated to reflect changes in the feeding ecology of the mussels on the short-term compared to the mantle (Shin et al., 2008; Redmond et al., 2010). Based on the multivariate analysis (NMDS) of the total FA composition, the mantle tissue was more segregated from the seston signature than the digestive gland. The outcome of the SIMPER analysis also underlined the larger difference in FA profile between the seston and the mantle (39.1%), than between the seston and the digestive gland (35.7%).

In this study, proportions of EPA and DHA were an average of five and fifteen times larger in the mantle than in the seston; and eight and eleven times greater in the digestive gland than in the seston, respectively. This difference was especially noticeable during autumn-winter for DHA, which had eighteen-fold and fourteen-fold higher amounts in the mantle and the gland than in the seston. The higher concentration of EPA and DHA in the tissues suggested a preferential incorporation of these FA in the mussels' tissues. A selective accumulation of PUFA and EPA was also noted in the tissues of four different bivalve species (Ventrella et al.,

2008; Ezgeta-Balić et al., 2012). Despite the fact bivalves have a limited ability for *de novo* synthesis of PUFA (Alkanani et al., 2007; Fernández-Reiriz et al., 2011), several invertebrates have been demonstrated to have elongase and desaturase enzymes capable of modifying dietary FA (Hall et al., 2006; Kelly and Scheibling, 2012; De Troch et al., 2012). De Troch et al. (2012) used a $^{13}$C labeled diet to demonstrate that copepods can increase their DHA levels from conversion of dietary 20:5ω3, even when 22:6ω3 was not present in the diet. Similarly, other invertebrates like sea urchin can convert 18:3ω3 to 20:5ω3, although the rate of conversion is slow (Bell et al., 2011). Nonetheless, previous work has demonstrated that clams have a very limited capacity to elongate and desaturate FA (Albentosa et al., 1996). Thus, it is probable that mussels had a limited capacity for the biosynthesis of EPA and DHA from precursor 18:3ω3 and that the enhanced levels of ω3 PUFA corresponded with selective retention of these FA.

In the present study, the highest proportion of DHA found in the mantle and gland could indicate a higher metabolic demand for FA with a structural function like DHA over FA with an energetic function like EPA (Freites et al., 2002 a; b). Results obtained by Khardin et al. (2003) with *Mytilus trossulus* fed different species of microalgae also suggested that a preferential absorption of ω3 PUFA takes place in the digestive gland, since the gland displayed a similar FA composition regardless of the FA signature of the diet. The results further showed that the PUFA content of the mantle and the digestive gland presented the highest values during the autumn-winter, while SAFA and MUFA were significantly higher in both organs during the warmer seasons. Ezgeta-Balić et al. (2012) also observed that the PUFA content of the digestive gland of *M. galloprovincialis* varied in an opposite fashion to the SAFA, concluding that the unsaturation degree of the FA increases during the colder seasons and decreases during the warmer seasons. This might be because high levels of PUFA are required for maintaining membrane fluidity of bivalves' cells during low water temperatures (Hall et al., 2002).

The FA composition of *M. galloprovincialis* feces was consistent to that observed for the same species by Frolov and Pankov (1995), who detected that 16:0 was the predominant FA in the feces (22.6-27.3%) and SAFA represented the most abundant class (43.4-51.0%) when mussels were maintained under laboratory conditions between 10 and 20 ºC. Khardin et al. (2003)

reported that the feces of *M. trossulus* collected under natural conditions contained high levels of 16:0 (23.3%) and SAFA (47.6%). Traditionally, studies investigating the trophic links among primary producers and bivalves have focused on the FA analysis of mussels' soft tissues and the particulate matter present in the water (Alkanani et al., 2007; Wong et al., 2008; Ezgeta-Balić et al., 2012). In this study, however, the analysis of the FA profile of the feces also provided a valuable insight on the seasonal changes occurring on the FA signature of the diet, highlighting which FA were assimilated, rejected or modified after assimilation of the diet (Kelly and Scheibling, 2012). SIMPER results showed that the seston and the feces had the closest resemblance in FA composition (22.3% dissimilarity) and were plotted within a short distance in the NMDS plot. This similarity in composition demonstrates that mussels recycle a large proportion of the FA contained in the seston through their biodeposits, returning a lot of the fatty acids back to the food web to be further exploited by other trophic levels. Overall, the fatty acid profile of the feces revealed a dominance of diatom and bacteria FA markers in the summer, whereas dinoflagellate markers were most important in the spring, autumn and winter. The intense upwelling of nutrient-rich waters increased the abundance of PUFA in the seston during spring, favoring polyunsaturated fatty acids to move up the trophic chain, from phytoplankton to mussels, as evidenced by the significantly higher levels of ω3 PUFA (i.e. EPA and DHA) in the feces. Likewise, a previous study noted that the FA profile of the feces of consumers like the crab *Parasesarma erythodactyla*, resembled the profile of their primary food source (Hall et al., 2006).

This study highlighted that the FA signatures of seston, mussels' organs and feces sampled at the rafts distant and close to the fish cages were very similar (Fig. 5). The results of the global NMDS plot and ANOSIM analysis showed that the FA composition of the fish feed was very different compared to the seston (51.2%), mussels' organs (53.4%) and feces (50.6%) (Fig. 5). Seston and mussels' organs and feces sampled 170 m from the red sea bream cages did not incorporate the FA signature of any fish feed particulate waste (i.e. from feed 'fines' up to larger particles <50 µm) that could be suspended in the seston. On the contrary, Gao et al. (2006) found that mussel *Perna viridis* cultured inside net-pens had significantly higher levels of trash

fish meal biomarker 18:1ω9 and a closer FA profile to the trash fish meal compared with mussels from the reference site. Handå et al. (2012a) identified an increase of 18:1ω9 in the mantle and digestive gland of *M. edulis* cultured 60 and 120 m away from salmon cages. The results of the former surveys, however, are not directly comparable to those of our study due to differences in experimental and sampling design, as mussels were suspended directly from the fish cages (Gao et al., 2006) or closer to the cages (Handå et al., 2012a) compared to this study (170 m; Fig. 1). Mussels may have been located too far to utilize uneaten feed particles in this study, as the majority of the particles are only available within 50 to 60 m from the fish cages (Cheshuk et al., 2003; Lander et al., 2013). However, the position of the rafts and fish cages in this study could not be altered as it was regulated through specific fish and shellfish management plans for coastal areas and did not obey a hypothetical optimal design for fish waste exploitation by the mussels.

Mussels grown in the vicinity of fish aquaculture sites might only consume feed pellets undigested particles as an alternative nutrient source when there is low availability of seston (Stirling and Okumus, 1995; Troell et al., 2003; Cheshuk et al., 2003; Neori et al., 2004; 2007; Both et al., 2012). Handå et al. (2012a) and Gao et al. (2006) suggested that the assimilation of waste pellet particles and trash fish particles, respectively, is seasonal because fish meal-derived FA biomarkers were only detected in the mantle and digestive gland of the mussels during winter, a period with a shortage of food supply, when chlorophyll-*a* levels reached their minimum concentrations. The present study showed no evidence of seasonal-driven fluctuations in the incorporation of feed biomarkers by the filter-feeders. The discrepancies found between the results of this study and those of Handå et al. (2012a) and Gao et al. (2006) may reside in the distance between fish and mussel culture units, different current speeds of the sites, stocking density of the fish farm, amount of effluents released by the fish farm, size-class distribution of the feed waste particles and time available to intercept the particles, seston characteristics, primary productivity and the bivalve biomass cultured near the fish cages (Cheshuk et al., 2003; Troell et al., 2011; Cranford et al., 2013). These constraints can limit the capacity of mussels to remove particulate organic fish waste in an open-water scenario like in this study (Cranford et

al., 2013). Mussels in the Galician Rías have a high supply of natural seston and chlorophyll that supports one of the highest growth rates in the world (Figueiras et al., 2002). Furthermore, studies on current velocities measured simultaneously by Zuñiga et al. (2014) confirmed that the rafts are located in an environment with fast current action that could disperse and dilute the feed undigested particles. In addition, mussels may have been restricted by a limited amount of fish uneaten feed particles, due to the low density of fish cultured in Lorbé relative to the large mussel standing stock.

In summary, our findings indicated that the temporal variations in the fatty acid composition of the seston were mirrored in the profile of the mussels' organs and feces. The diet of *M. galloprovincialis* changed seasonally; there was a predominance of diatom-derived fatty acid biomarkers 16:1ω7, 20:5ω3 and 20:5ω3/22:6ω3>1 in the mussel tissues during the upwelling period in spring-summer, whereas dinoflagellate-specific FA 16:0, 18:1ω9, 18:4ω3, 22:6ω3 and 22:6ω3/20:5ω3>1 were the main dietary source during downwelling in autumn-winter. Bacteria also represented an additional input for the bivalves, especially during spring-summer, when bacterial markers 15:0, 17:0, 18:1ω7 and 18:1ω7/ 18:1ω9>1 were most abundant. The assimilation of these FA was further reflected in the biodeposits. Diatom and bacteria FA biomarkers predominated in the feces in the summer, whereas dinoflagellate markers were most important in the spring, autumn and winter. There was no evidence of any seasonal or spatial incorporation of fish feed FA markers in the seston, mussels' tissues or feces in this study.
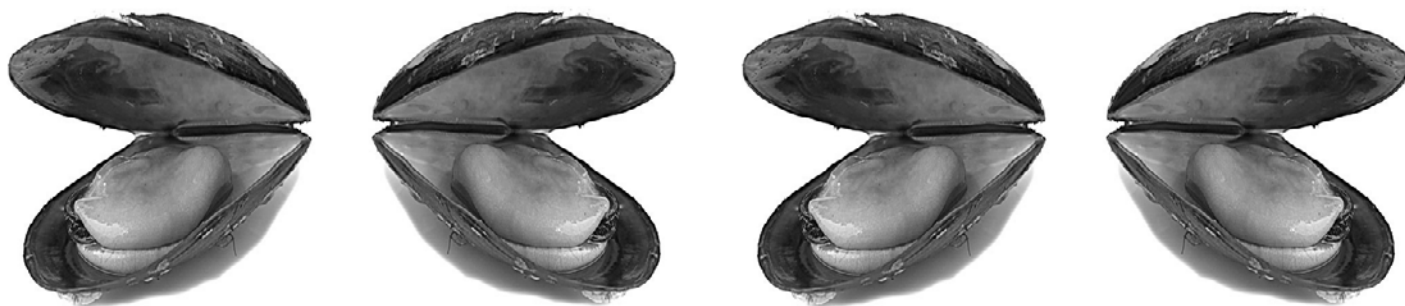
# Chapter 6

## Availability and utilization of waste fish feed by mussels *Mytilus edulis* in a commercial Integrated Multi-Trophic Aquaculture (IMTA) system: a multi-indicator assessment approach

# ABSTRACT

Fish feed waste enhancement of the particulate food supply and performance of mussels *Mytilus edulis* suspended near salmon cages at an Integrated Multi-Trophic Aquaculture (IMTA) site was assessed using a multi-indicator approach. Dietary indicators included bulk measurements of seston quantity and nutritional quality, proximate analysis (PA), fatty acid (FA) and stable isotope (SI) composition. Mussel tissue indicators consisted of PA and FA composition. Mussel performance was assessed from physiological integrations (Scope for Growth, SFG), growth efficiency ($K_2$) and condition index (CI). All measurements were made over 2 days at a commercial IMTA farm and a monoculture mussel farm in the Bay of Fundy (Canada). Significant differences detected in seston quantity and quality were within the range of natural spatial variability. The SFG of IMTA mussels was lower (28.71 J h$^{-1}$) than monoculture mussels (38.71 J h$^{-1}$) and reflected site differences in natural food availability and composition that affected absorption rate. PA of mussel organs didn't reflect a significant fish feed contribution to the mussel diet. However, dietary enhancement and assimilation of fish feed waste was demonstrated by significantly higher levels of feed FA biomarkers 20:1ω9, 18:2ω6, 18:1ω9 and low ω3/ω6 ratio in seston, mussel tissues and feces at the IMTA site than at the mussel farm. SI ($\delta^{13}$C and $\delta^{15}$N) in seston and mussel feces significantly differed among sites and IMTA mussels had significantly higher CI (21%) than monoculture individuals (16%). It was concluded that bulk indicators of the diet, short-term physiological integrations, and PA of mussel tissues have a limited capacity to detect dietary enhancement at IMTA sites. FA and SI tracers of fish feed waste were shown to be more sensitive for detecting the low-levels of diet enhancement within the large range of natural seston variation.

**Key words:** *Integrated Multi-Trophic Aquaculture; mussels; Scope for Growth; biochemical composition; fatty acid biomarkers; condition index*

# 1. Introduction

Salmon monoculture discharges large amounts of solid effluents that include fish feed waste (i.e. small feed 'fines' and larger uneaten feed particles) and feces, as well as dissolved nutrients like excretory products. Fish wastes can generate eutrophication of the water column (Naylor et al., 2000; Wang et al., 2012) and affect the benthic environment beneath the net-pens (Folke et al., 1994; Pohle et al., 2000). Canada is the world's fourth largest producer of Atlantic salmon (*Salmo salar*) after Norway, U.K. and Chile, with 101,385 tons farmed in 2010 (FAO, 2012). The Passamaquoddy Bay in New Brunswick is the primary region of salmon farming within the Bay of Fundy and produces 25,000 tons year$^{-1}$ worth 159 million USD ($) (Statistics Canada, 2010). Presently, the blue mussel *Mytilus edulis* is being utilized for partial biomitigation of fish wastes by means of Integrated Multi-Trophic Aquaculture (IMTA) in the Bay of Fundy.

In brief, IMTA is a practice in which organic and inorganic wastes from fed aquaculture species (e.g. finfish) are assimilated by organic extractive species (e.g. mussels, sea cucumbers, sea urchins) and inorganic extractive species (e.g. seaweed) that are cultured alongside the fed aquaculture species (Neori et al., 2004; MacDonald et al., 2011; Nelson et al., 2012). Mussels are cosmopolitan species and general suspension-feeders that are cultured in dense aggregations. The large biofiltration capacity of suspended mussels has provided rationale for their use in IMTA systems (Cranford et al., 2013). In addition, mussels are valuable sources of proteins and essential ω3 polyunsaturated fatty acids (PUFA) like 20:5ω3 (eicosapentaenoic acid, EPA) and 22:6ω3 (docosahexaenoic acid, DHA), beneficial for healthy human development and prevention of diseases (Fernández-Reiriz et al., 1996; Orban et al., 2002). Despite the importance of examining the ecophysiological components of mussel growth (Scope for Growth; SFG: integration of clearance, ingestion, absorption, respiration and excretion rates) in IMTA systems, studies on physiological rates are scarce and have mainly focused on the absorption efficiency and clearance rate (Reid et al., 2010; MacDonald et al., 2011; Irisarri et al., 2013). Even if previous studies in the Bay of Fundy reported increased absorption efficiencies for *M. edulis* cultured within a few meters from salmon cages or exposed to fish

effluents under laboratory conditions (Reid et al., 2008; 2010; MacDonald et al., 2011; Lander et al., 2012), they did not evidence if the enhanced absorption ultimately resulted in a greater Scope for Growth. The utilization of fish feed by mussels has been further studied with other ecophysiological and biochemical indicators like the condition index (CI) (Taylor et al., 1992; Cheshuk et al., 2003; Peharda et al., 2007; Lander et al., 2012) and the proximate analysis (PA; protein, carbohydrate and lipid tissue content) (Taylor et al., 1992; Both et al., 2011; 2012; 2013). However, given that mussels cultured in open-water IMTA systems are exposed to a mixture of seston supplemented with fish wastes, it still remains challenging to elucidate if the augmented absorption efficiency, CI or proximate composition owes to the utilization of fish feed waste or simply corresponds to increases in seston loads within the range of seasonal and spatial variations that occur at each site. This challenge might be overcome by using a multi-indicator approach, as the information provided by physiological (SFG) and biochemical (PA) indicators can be complemented with the results provided by molecular indicators like fatty acids (FA) and stable isotopes (SI). FA and SI have been effectively used for investigating seasonal and spatial changes in a consumer's diet on the long-term (Alfaro et al., 2006; Allan et al., 2010; Guest et al., 2010; Zhao et al., 2013). Fish feed contains specific FA (18:1ω9, 18:2ω6, 20:1ω9, 20:4ω6, 20:1ω11 and 22:1ω11) that can be transferred and conserved along the trophic chain and used as biomarkers to indicate their consumption by suspension-feeders (Gao et al., 2006; Redmond et al., 2010; Both et al., 2012; 2013; Handå et al., 2012 a;b). The mussel diet can be further inferred from the relative percentages of FA biomarkers characteristic of diatoms (16:1ω7, 20:5ω3, 16:1ω7/16:0>1, 20:5ω3/22:ω3>1), dinoflagellates (16:0, 18:1ω9, 18:4ω3, 22:6ω3 and 22:6ω3/20:5ω3>1) (Budge et al. 2001; Dalsgaard et al. 2003, Shin et al., 2008; Kelly and Scheibling, 2012) and bacteria (15:0, 17:0, 18:1ω7, 18:1ω7/18:1ω9<1) that colonize the suspended particulate material (Bachok et al., 2003; Xu and Yang, 2007; Allan et al., 2010). The carbon ($\delta^{13}$C) and nitrogen ($\delta^{15}$N) isotopes of the consumer reflect the isotopic signature of the diet, although the consumer is usually enriched by 1‰ in $\delta^{13}$C with respect to its diet and by 3-4 ‰ in $\delta^{15}$N with each trophic level, since lighter isotopes ($^{12}$C and $^{14}$N) are usually lost through metabolism or fractionation during synthesis of tissues (Michener and Kaufman, 2007).

This study aimed to investigate the potential utilization of fish feed particulate waste by mussels *Mytilus edulis* cultured in a salmon IMTA system in the Passamaquoddy region of the Bay of Fundy. The utilization of fish feed waste was assessed using a multi-indicator approach, to evaluate if co-cultured mussels enhanced their performance at the ecophysiological (Scope for Growth and condition index) biochemical (proximate composition) and molecular (FA, SI) levels of biological organization. These indicators were compared to those measured in mussels cultivated at a monoculture farm.

## 2. Material and methods

### 2.1. Experimental site

The study was performed at two sites within Passamaquoddy Bay, in the Bay of Fundy (Fig. 1). The IMTA site was sampled the morning of the 14th June 2011 -when salmon were being fed- and consisted of a commercial salmon farm in Clam Cove, on the west side of Deer Island (44°57'52.2''N; 67°0'46.65''W; Fig. 1). Salmon (*Salmo salar*) were cultured in 16 net-pens (100 m circumference plastic polar circle cages in a 4x4 array). Four commercial mussel rafts were moored adjacent to the salmon cage array. Each raft consisted of 4 concentric circles of flotation pipe (outside ring = 100 m circumference) from which mussels were hung with 7 m long socks at a density of ~1,000 mussels m$^{-1}$. The IMTA site is located in shallow waters with low current velocity (10 m depth and 5 cm s$^{-1}$, respectively) (Chang and Page, 2011).

The monoculture mussel farm was visited on the following day. It was a commercial salmon farm (Lease MF 377; 45°02'58.2''N; 67°01'55.43''W; Fig. 1) that was being fallowed during the normal 3-year culture cycle and was chosen due its proximity to the IMTA site (8.5 km away). Mussels were hung from the flotation collars of the cages in 3 m long socks with 800 mussels m$^{-1}$. The monoculture is situated in deeper waters with high current velocity (20-25 m depth and 10-15 cm s$^{-1}$, respectively) (Chang and Page, 2011).
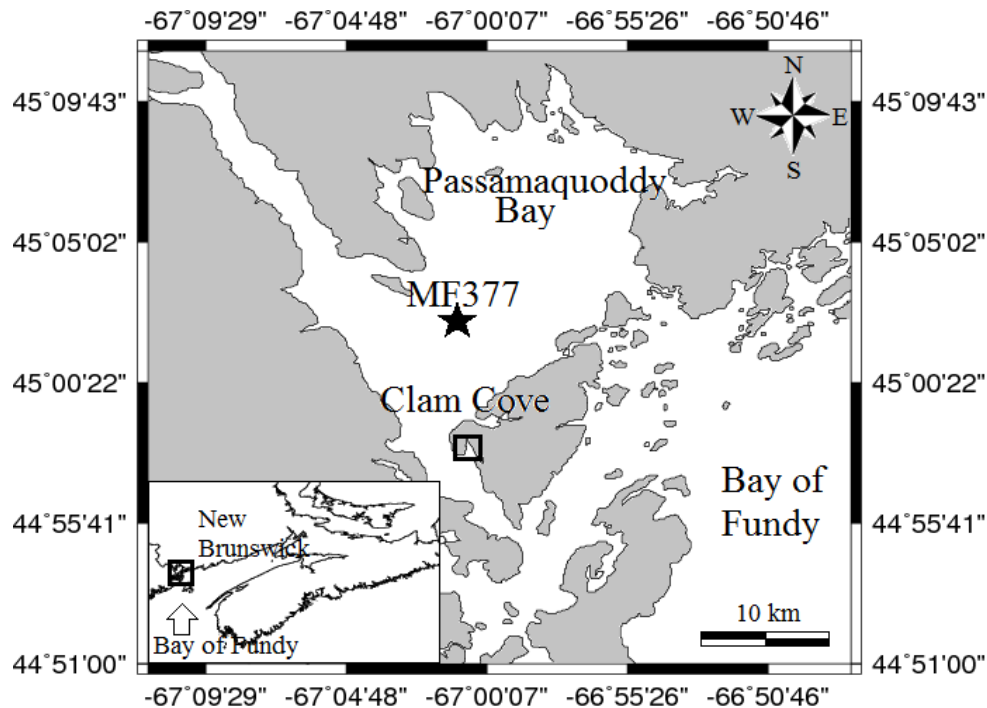
Fig.1. Map of the Passamaquoddy region of the Bay of Fundy (S.W. New Brunswick, Canada) showing the location of the IMTA site in Clam Cove (44°57'52.2''N; 67°0'46.65''W) and the monoculture farm MF377 (45°02'58.2''N; 67°01'55.43''W).

## 2.2. Field sampling protocol

Mussels with 50-60 mm shell length were collected from the IMTA and monoculture sites for physiological (n=10 to 30 mussels per site for each physiological determination), proximate composition (n=3 per site), fatty acid (n=3 per site) and condition index (n=23 per site) determinations. In the case of PA and FA composition, each replicate consisted of the mantle tissue or the digestive gland of 3-5 mussels.

Physiological rates were determined onboard a moored ship to preserve natural physico-chemical conditions and food availability. Mussels were cleaned of epibionts and placed in chambers with flowing seawater. Seawater from 3 m depth was supplied by a peristaltic pump to a header tank and filtered through a 50 µm before being distributed at a 3 l min$^{-1}$ (Filgueira et al., 2006). Seawater (n=3) was collected from a empty chamber during the physiological

determinations to analyze the chlorophyll-*a*, proximate composition (PA), fatty acids (FA) and stable isotopes (SI) of the seston. Seawater was filtered through pre-washed and pre-combusted Whatman GF/F filters and rinsed with isotonic ammonium formate. The total particulate matter (TPM, mg $l^{-1}$), organic and inorganic fractions (POM and PIM, mg $l^{-1}$) and quality (*f*=POM/TPM) of the seston were characterized in a parallel survey (Irisarri et al., 2013).

Mussel feces (n=6 per site; each replicate comprised feces from 12 mussels) were collected after placing bivalves in a 19 l mesocosm. The mesocosm consisted of three replicate tanks, each divided into 16 compartments containing 3-4 mussels (Filgueira et al., 2006). Feces were collected 3 to 4 h after being harvested and filtered as the seston samples for PA, FA and SI signatures. Mussels selected for PA and FA determinations were dissected to separate the mantle tissue from the digestive gland. Lastly, three replicates of 150-300 mg of fish feed were collected directly from the feed barge at the IMTA site for PA, FA and SI determinations.

## 2.3. Seston analysis

Chlorophyll *a* was extracted with acetone and quantified using a Perkin-Eimer Lambda 35 UV/VIS spectrophotometer following Jeffrey and Humphrey (1975). The total particulate matter concentration (TPM; mg $l^{-1}$) was calculated after drying the filters at 110ºC for 12 h until constant weight. The difference between the dry weight and ashed weight (450ºC for 3h) of the filters indicated the organic mass, which was used to calculate particulate organic matter concentration (POM; mg $l^{-1}$) and the organic fraction (*f*=POM/TPM). Samples of mussels' organs and feces placed in pre-weighed aluminum pans were analyzed for organic content in a similar way.

## 2.4. Proximate (PA) and fatty acid (FA) analyses

Samples were stored at -50 °C until lyophilization with a freeze-dryer (Ilshin Lab Co. Ltd, Korea) and further stored at -20 °C until homogenization with a mortar and a pestle or with an

ultrasonic Branson Sonifier (250/450 USA) in the case of the organs. Three subsamples of each replicate were used for protein, carbohydrate, glycogen and lipid determinations.

Protein were determined following Lowry et al. (1951), after alkaline hydrolysis with 0.5 N NaOH at 30°C for 24 h. Carbohydrates were quantified according to the phenolsulphuric acid method (Strickland and Parsons, 1968) using glucose as the standard. Glycogen was estimated in the same way, after sample precipitation with 100 % ethanol. Lipids were extracted according to Bligh and Dyer (1959) method modified by Fernández-Reiriz et al. (1989). Total lipids were colorimetrically quantified following Marsh and Weinstein (1966) with a tripalmitin standard (Sigma Aldrich Inc., Buchs, Switzerland). Results for each biochemical component were expressed as percent of dry weight (%DW) and as energetic content in terms of Jules $l^{-1}$ (seston) or Jules $mg^{-1}$ (fish feed and mussel samples). Energy conversion factors for proteins (18.00 J $mg^{-1}$), lipid (35.24 J $mg^{-1}$) and carbohydrate (17.16 J $mg^{-1}$) were obtained from Beukema and De Bruin (1979).

The fatty acids (FA) from the total lipids were transesterified to FA methyl esters (FAME) with a solution of toluene and concentrated sulfuric acid solution in methanol (1.5:100 ml) according to Christie (1982). FAME were injected in a gas chromatographer (Perkin-Elmer, 8500) equipped with a flame ionization detector and a 30 m capillary column of flexible silica (Supelco, SP-2330). Nitrogen was used as the carrier gas at a pressure of 0.069 Pa. The injector was programmed at 275 ºC and operated in solvent elimination mode (Medina et al., 1994). The column increased from 140°C to 210°C at a rate of 1°C $min^{-1}$.

FAME obtained for mussel feces were purified by adsorption chromatography before being injected in the chromatographer, eluting the samples with a mobile phase consisting of a mixture of hexane and ether (95:5 ml) following Christie (1982). Purified samples were evaporated to dryness under a stream of nitrogen and resuspended with toluene.

Fatty acids were identified by co-injection of the samples along standard mixtures of nonadecanoic acid (19:0) to compare the relative retention times. Results for each FA were expressed as the relative percentage (%) of the total FA content ± standard deviation.

## 2.5. Stable Isotopes (SI) analysis

Seston and mussel feces were filtered onto Whatman GF/F filters and rinsed with isotonic ammonium formate, dried at 60 °C for 24 h, pulverized and placed in individual covered petri dishes. Fish feed was also dried and pulverized before being placed into sealed containers. Blank filters were run as a control. Carbon and nitrogen SI ratios were determined using a Carlo Erba NC 2500 Elemental Analyzer (Milan, Italy) coupled to a Finnegan Delta mass spectrometer (Breman, Germany) via continuous flow.

Isotopic values were expressed in standard δ-unit notation, in units of parts per thousands (‰), according to the following equation:

$$\delta X = [(R_{sample}/ R_{standard}) -1] \times 10^3 \tag{1}$$

where $X = {}^{13}C$ or ${}^{15}N$, and R is either ${}^{13}C:{}^{12}C$ ratio for carbon or ${}^{15}N:{}^{14}N$ ratio for nitrogen of the sample and standard, respectively. The values were reported relative to the Vienna Pee Dee Belemnite standard for carbon and to atmospheric $N_2$ for nitrogen. Isotope values were normalized using nicotinamide, cornmeal, aquatic moss, spirulina and ephedra plant as secondary standards (Stable Isotopes Nature Laboratory, SINLAB). All of these standards were calibrated against International Atomic Agency (IAEA) standards. Precision of the results was established by replicate measurement of selected sub-samples.

## 2.6. Mussel physiological measurements

Clearance rates (CR, $l\,h^{-1}$) were estimated from the reduction in suspended particles between the water surrounding the mussels and the outflow of the chambers (Filgueira et al., 2006). The organic ingestion rate (OIR, mg $h^{-1}$) was estimated as the product of CR and the POM. Absorption rate (AR, mg $h^{-1}$) was calculated as the product of the absorption efficiency (AE; see Irisarri et al., 2013) and the OIR. Respiration rate (VO$_2$, ml $h^{-1}$) and ammonia excretion rate (VNH$_4$-N, µg $h^{-1}$) were determined as the difference in oxygen and ammonia concentrations between the control and experimental chambers as in Fernández-Reiriz et al. (2012). The ratio of oxygen consumed to nitrogen excreted (O:N) was calculated in term of atomic equivalents

(Widdows, 1985). All physiological rates were standardized for an individual of 60 mm for the CR and for an individual of 1 g tissue dry weight for both $VO_2$ and $VNH_4$-N. The Scope for Growth (SFG, J h$^{-1}$) was computed following the energy balance equation proposed by Winberg (1960) and Ivlev (1966). Net growth efficiency ($K_2$) was calculated as SFG/AR.

## 2.7. Condition index (CI)

The body tissues and the shell were dried separately at 110°C for 24 h. The dry shell weight ($DW_{shell}$) and dry tissue weight ($DW_{tissue}$) were determined to calculate the condition index (Freeman, 1974): CI = ($DW_{tissue}$/$DW_{shell}$) x 100.

## 2.8. Statistical analysis

The effect of the location on the proximate composition, stable isotopes, physiological rates and condition index were tested using a one-way analysis of variance (ANOVA) followed by a Tukey's HSD test. Assumptions of normality and homoscedasticity were checked with Shapiro-Wilk and Levene test. A ranked ANOVA (non-parametric) was performed when data didn't fit these assumptions. Statistical analyses were carried out using STATISTICA 7.0 software (StatSoft Inc).

The fatty acid (FA) percentage of the samples was logarithmically (log (x+1)) transformed and a Bray-Curtis similarity matrix was constructed to segregate the data in a 2-D non-metric multidimensional scaling plots (NMDS) with PRIMER 6.0 software (Clarke and Warwick, 1994). Stress values <0.05 indicated an excellent representation of the clusters and <0.1 and <0.2 indicated good and potentially useful plots, respectively. One-way analysis of similarity (ANOSIM) tested for spatial differences among the FA composition of IMTA and monoculture samples and compared the overall FA profile of the fish feed with that of the seston, mussels' organs and feces. Values of the *R* statistic of ANOSIM close to 1 indicated well separated clusters; while values close to 0 indicated a weak separation of the clusters. ANOSIM was

followed by a similarity percentage analysis (SIMPER) which indicated the FA contributing to the highest percentages of the observed dissimilarities between samples. One-way ANOVA followed by Tukey's HSD tests was conducted to identify differences in specific FA biomarkers and FA classes among samples.

# 3. Results

## 3.1. Seston composition

One-way ANOVA followed by *post-hoc* testing showed 33.5 % higher chl-*a* concentrations at the IMTA site compared with the monoculture farm ($F_{(1,4)}$=15.43, $P$<0.05; Table 1). In contrast, the POM, organic fraction (*f*), proteins, carbohydrates and lipids contained in the seston at the monoculture were significantly higher than at the IMTA site (Tables 1 and 2; Fig. 2). Thus, seston had more than two-fold higher energetic content at the monoculture in terms of proteins ($F_{(1,4)}$=129.41, $P$<0.001), carbohydrates ($F_{(1,4)}$=55.66, $P$<0.001) and lipid content ($F_{(1,4)}$=355.16, $P$<0.001) (Table 1).

Seston was constituted mainly by FA 16:0, 16:1ω7 and 18:1ω9, while saturated (SAFA) and monounsaturated (MUFA) fatty acids were present in higher proportions than polyunsaturated fatty acids (PUFA) (Table 3). The NMDS plot showed a strong segregation between the FA profile of the IMTA seston samples and the monoculture site (Fig. 3a, stress 0.01). This was confirmed by the high global $R$ value obtained in the one-way ANOSIM (global $R$: 0.556, $P$=0.1). The average spatial dissimilarity for the seston composition was 8.3 % and 20:4ω6, 18:0 and 20:1ω9 were the main FA contributing to these differences (SIMPER, 16.2, 12.0, 8.8 %, respectively) (Fig. 4a).

ANOVA showed significantly higher levels of fish feed FA biomarkers 20:1ω9 ($F_{(1,4)}$=23.37, $P$<0.01) and 18:2ω6 ($F_{(1,4)}$=11.26, $P$<0.05) in the seston collected at the IMTA farm (Table 3; Fig. 5a). In contrast, fish feed marker 20:4ω6 showed higher levels in seston from the monoculture site. Seston from both sites was characterized by a predominance of diatom

markers 16:1ω7 and EPA and a high EPA/DHA ratio (2.20) in comparison to dinoflagellate-specific biomarkers (16:0, 18:1ω9, 18:4ω3, DHA, DHA/EPA=0.45) (Fig. 5b). The contribution of bacteria FA biomarkers (15:0, 17:0, 18:1ω7) and the bacteria ratio 18:1ω7/18:1ω9 (0.3) was very low (Table 3; Fig. 5b).
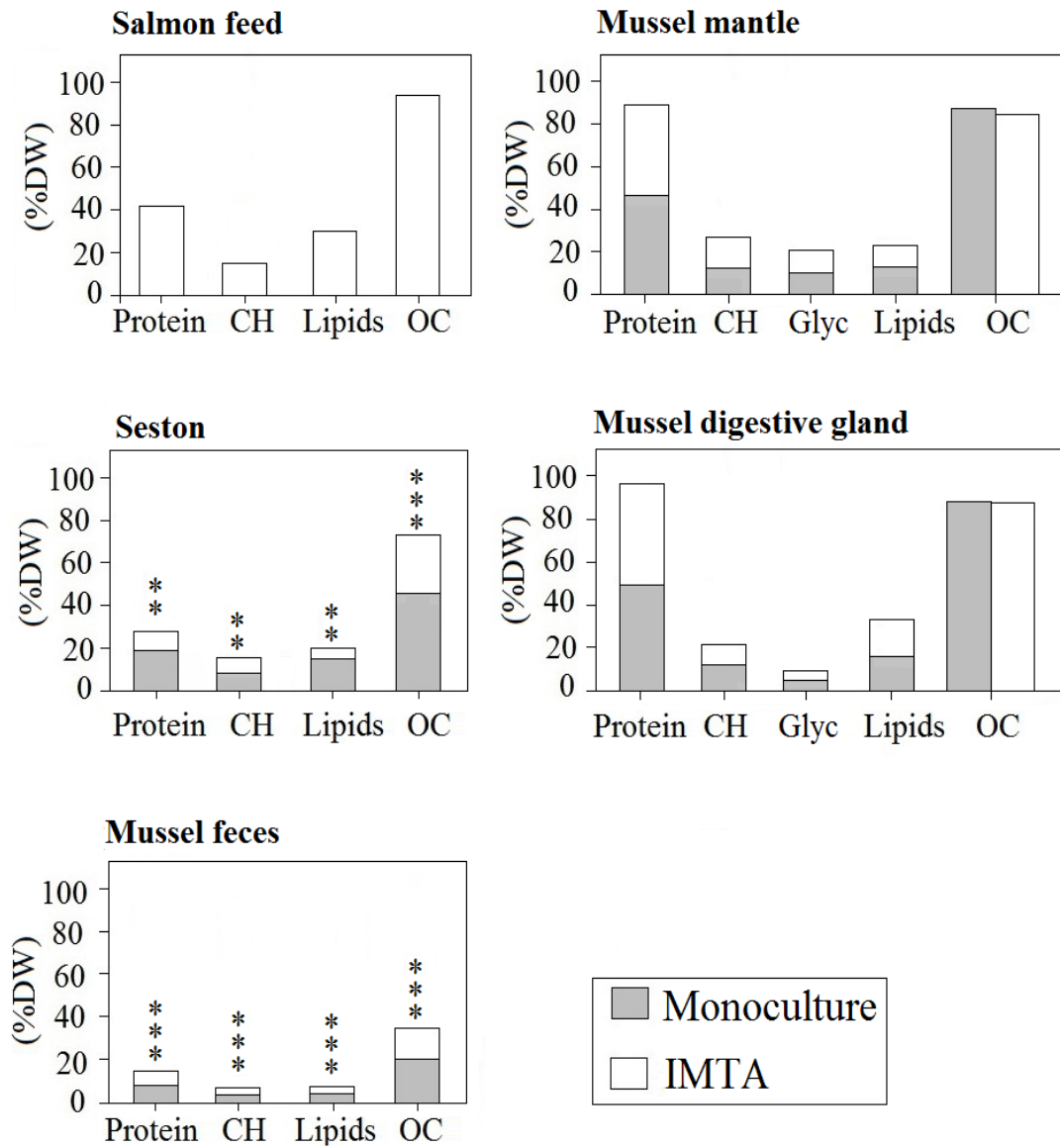


Fig. 2. Biochemical composition (% dry weight; %DW) of the fish feed, seston, mussels' organs and feces (n=3 for each sample) obtained at the monoculture mussel farm (grey bars) and the IMTA station (white bars) in the Bay of Fundy. Significant differences are indicated with **$P<0.01$ and ***$P<0.001$. CH= carbohydrates, Glyc=glycogen, OC=organic content.

| Sites | Chl-a µg l$^{-1}$ | POM mg l$^{-1}$ | $f$ (POM/TPM) | Proteins % DW | J l$^{-1}$ | Carbohydrates % DW | J l$^{-1}$ | Lipids % DW | J l$^{-1}$ | Total energy J l$^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Monoculture | 2.50 ± 0.46 | 0.75 ± 0.03*** | 0.45 ± 0.02*** | 18.80 ± 2.30** | 5.67 ± 0.54*** | 8.47 ± 1.13** | 2.43 ± 0.24*** | 14.41 ± 0.63** | 8.55 ± 0.83*** | 16.65 ± 1.30 *** |
| IMTA | 3.76 ± 0.06* | 0.54 ± 0.01 | 0.27 ± 0.01 | 8.91 ± 2.07 | 3.17 ± 0.55 | 4.30 ± 1.19 | 1.46 ± 0.31 | 4.62 ± 0.09 | 3.24 ± 0.18 | 7.87 ± 0.85 |

Table 1. Chlorophyll content, gravimetric and biochemical characterization of the diet at the monoculture site and the IMTA farm. Values are mean ± SD. Statistical differences within the same column are indicated by *$P$<0.05, **$P$<0.01, ***$P$<0.001. Values for particulate organic matter (POM) and organic fraction ($f$) were simultaneously determined in Irisarri et al. (2013).
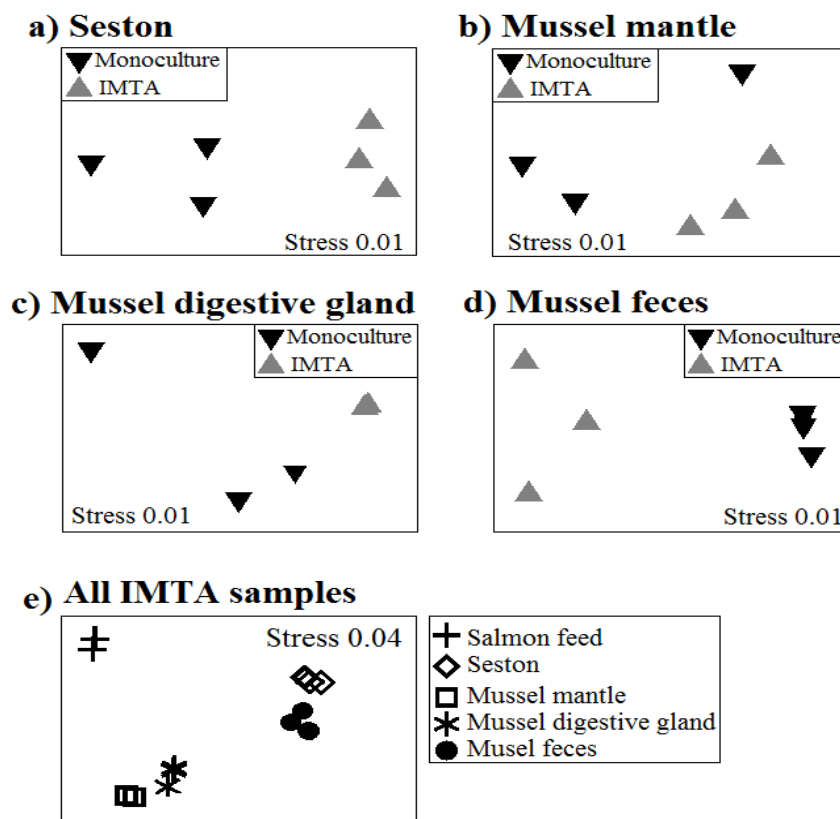


Fig. 3. Non-metric multidimensional scaling (NMDS) plot based on logarithmically transformed fatty acid composition of: a) seston, b) mantle, c) digestive gland, d) feces sampled at the monoculture site and the IMTA station and f) all the mussel samples and fish feed obtained at the IMTA station. The stress values measure the correlation between the Euclidian and physical distance calculated between samples: 0.05 (excellent correlation), 0.1 (good correlation) and 0.2 (weak correlation).

| | Biochemical composition | | | | FA classes | | |
|---|---|---|---|---|---|---|---|
| | SS | *F*-value (1,4) | *P*-value | | SS | *F*-value (1,4) | *P*-value |
| *Seston* | | | | *Seston* | | | |
| *f* | 0.14 | 430.97 | <0.001*** | SAFA | 13.50 | 13.50 | <0.05* |
| Ash | 469.33 | 138.34 | <0.001*** | MUFA | 1.50 | 0.37 | 0.57 ns |
| Protein | 146.76 | 30.56 | <0.01** | PUFA | 121.99 | 0.79 | 0.42 ns |
| CH | 26.12 | 19.45 | <0.01** | DMA | 0.01 | 0.81 | 0.41 ns |
| Glycogen | - | - | - | ω3/ ω6 | 0.16 | 0.03 | 0.85 ns |
| Lipids | 143.82 | 706.48 | <0.01** | NMI | 0.06 | 20.45 | <0.01** |
| | | | | | | | |
| *Mantle* | | | | *Mantle* | | | |
| OC | 8.96 | 4.93 | 0.09 ns | SAFA | 0.38 | 0.009 | 0.77 ns |
| Ash | 8.96 | 4.93 | 0.09 ns | MUFA | 11.44 | 0.49 | 0.51 ns |
| Protein | 10.71 | 1.63 | 0.27 ns | PUFA | 7.35 | 0.21 | 0.66 ns |
| CH | 8.92 | 2.44 | 0.19 ns | DMA | 0.002 | 0.01 | 0.91 ns |
| Glycogen | 2.70 | 0.57 | 0.49 ns | ω3/ ω6 | 1.34 | 18.44 | <0.001*** |
| Lipids | 4.01 | 0.62 | 0.47 ns | NMI | 0.22 | 0.39 | 0.56 ns |
| | | | | | | | |
| *Gland* | | | | *Gland* | | | |
| OC | 0.01 | 0.09 | 0.77 ns | SAFA | 1.5 | 0.37 | 0.57 ns |
| Ash | 0.01 | 0.09 | 0.77 ns | MUFA | 0.16 | 0.03 | 0.85 ns |
| Protein | 0.03 | 0.00 | 0.96 ns | PUFA | 1.5 | 0.37 | 0.57 ns |
| CH | 0.05 | 0.22 | 0.66 ns | DMA | 0.94 | 2.15 | 0.21 ns |
| Glycogen | 0.12 | 0.42 | 0.55 ns | ω3/ ω6 | 1.5 | 0.37 | 0.57 ns |
| Lipids | 0.83 | 1.69 | 0.26 ns | NMI | 0.76 | 0.86 | 0.40 ns |
| | | | | | | | |
| *Feces* | | | | *Feces* | | | |
| OC | 83.80 | 32.29 | <0.001** | SAFA | 20.98 | 22.9 | <0.01** |
| Ash | 81.12 | 30.61 | <0.001** | MUFA | 38.85 | 135.84 | <0.001*** |
| Protein | 5.62 | 48.24 | <0.001** | PUFA | 2.97 | 1.61 | 0.27ns |
| CH | 0.99 | 22.97 | <0.001** | DMA | 0.005 | 0.558 | 0.49 ns |
| Glycogen | - | - | - | ω3/ ω6 | 0.73 | 5.61 | 0.07 ns |
| Lipids | 1.05 | 19.34 | <0.001** | NMI | 0.02 | 0.35 | 0.58 ns |

Table 2. Results of the one-way ANOVA testing for significant differences between the organic fraction (*f*), biochemical composition (%DW) and fatty acid (FA) classes of the seston, mantle tissue, mussel digestive gland and feces of *M. edulis* sampled at the monoculture and the IMTA sites. Significant levels are denoted as *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ns (not significant).

### 3.2. Mussel tissues proximate and fatty acid composition

ANOVA indicated no site-specific differences in the OC and PA of the mantle tissue when values were expressed as %DW ($P>0.05$; Table 2; Fig. 2). Thus, there were no significant differences detected for the protein ($F_{(1,4)}=0.73$, $P=0.44$), carbohydrates ($F_{(1,4)}=2.91$, $P=0.16$) and lipid ($F_{(1,4)}=0.43$, $P=0.54$) energetic content at both sites (Table 4). Eicosapentaenoic acid (EPA, 20:5$\omega$3), 16:0 and docosahexaenoic acid (DHA, 22:6$\omega$3) were the most abundant FA in the mantle tissue, while PUFA predominated over SAFA and MUFA (Table 3). The NMDS plot showed that the FA profile of the mantle differed slightly in composition among sites (Fig. 3b, stress 0.01, one-way ANOSIM, global $R$: 0.111, $P=0.4$). Average dissimilarity between sites was 6.15 %. Spatial dissimilarity was principally attributed to 16:1$\omega$7 and the fish feed biomarkers 18:1$\omega$9 and 18:2$\omega$6 (SIMPER, 8.4, 7.3 and 6.2%, respectively) (Fig. 4b). The mantle of mussels cultured at the IMTA site had 40 % higher content of feed biomarker 18:2$\omega$6 than mussels at the monoculture ($F_{(1,4)}=129.32$, $P<0.001$; Fig. 5a). In contrast, fish feed marker 18:1$\omega$9 showed slightly higher levels in the mantle tissue from the monoculture site. The fish feed ratio $\omega$3/$\omega$6 also proved to be higher in the mantle of mussels from the monoculture site (Tukey HSD, $P<0.001$; Fig. 5a; Table 2). The mantle tissue accumulated high amounts of the diatom biomarkers 16:1$\omega$7 and EPA and a high EPA/DHA ratio (2.40), while trophic biomarkers for dinoflagellates and bacteria were present in a lower proportion (Fig. 5b).

The PA of the digestive gland resembled that of the mantle and there were also no spatial differences in the OC and % DW of the biochemical components of the gland ($P>0.05$, Table 5; Fig. 2). Concurrently, the gland showed similar energy content at both sites, with no statistical differences detected for the protein ($F_{(1,4)}=0.01$, $P=0.94$), carbohydrates ($F_{(1,4)}=0.19$; $P=0.68$) and lipid ($F_{(1,4)}=1.94$; $P=0.23$) energetic content among sites (Table 2). EPA, 16:0 and 16:1$\omega$7 were the most abundant FA in the digestive gland, while PUFA predominated over SAFA and MUFA (Table 3). The NMDS plot showed a clear spatial separation between the FA profile of the mussel digestive gland (Fig. 3c, stress 0.01, one-way ANOSIM, global $R$: 0.481, $P=0.1$). The average dissimilarity between sites was 8.46 % and was due to fish feed biomarkers

18:1ω9, 20:1ω9 and 18:2ω6 (SIMPER, 12.5, 6.3 and 5.9 %) (Fig. 4c). These biomarkers showed significantly higher contents in the digestive gland of IMTA mussels ($F_{(1,4)}$=73.89; $F_{(1,4)}$=14.58; $F_{(1,4)}$=129.32, respectively; $P$<0.001) (Fig. 5a). The digestive gland of mussels from both sites presented high levels of diatom biomarkers, while FA biomarkers for dinoflagellates and bacteria were less abundant (Table 3; Fig. 5b).
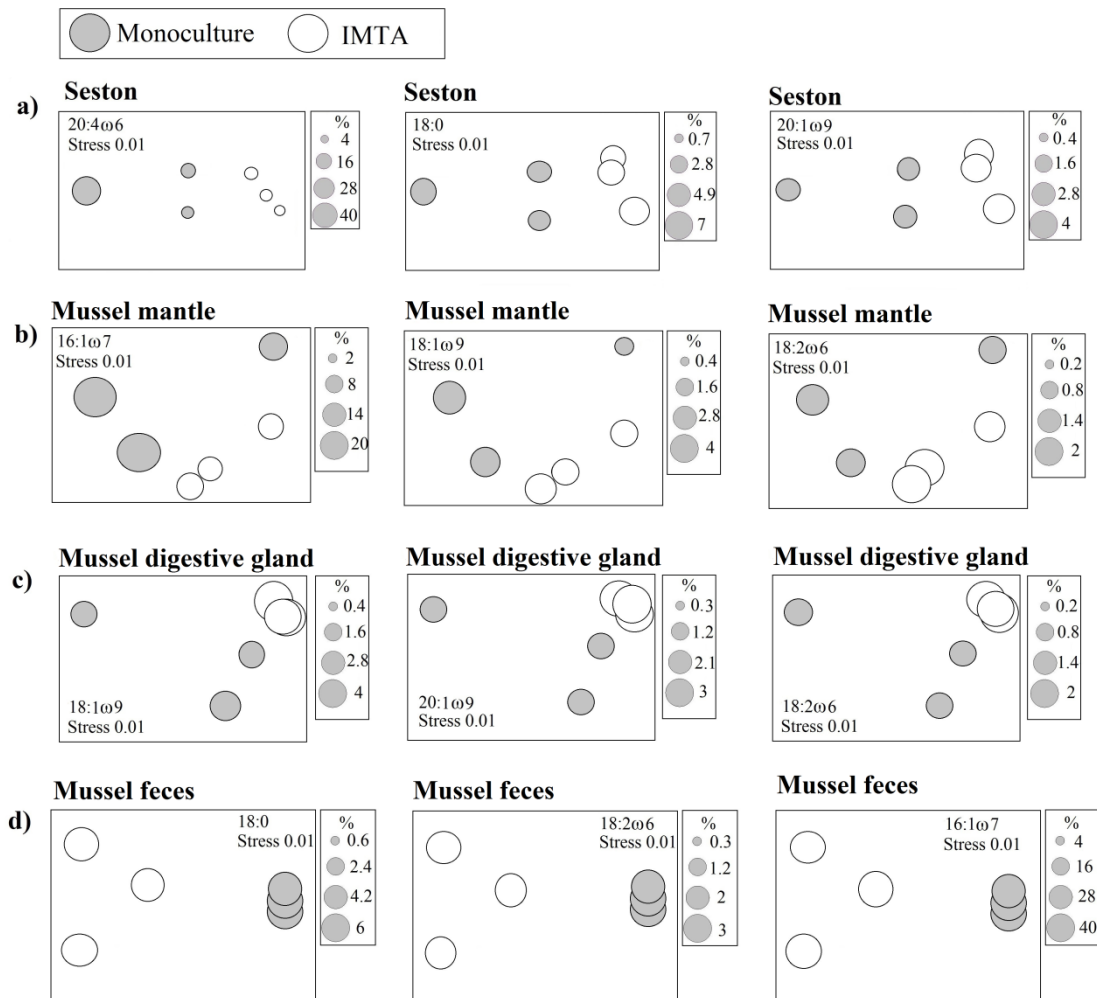


Fig. 4. Bubble plots representing the fatty acids that explained the highest percentage dissimilarity (SIMPER) between: a) seston, b) mantle, c) digestive gland and d) feces sampled at the monoculture and the IMTA station.

| FA | Fish feed | Seston | | Mantle | | Digestive gland | | Feces | |
|---|---|---|---|---|---|---|---|---|---|
| | | Monoculture | IMTA | Monoculture | IMTA | Monoculture | IMTA | Monoculture | IMTA |
| C 14:0 | 4.08 ± 0.17 | 9.02 ± 2.62 | 10.11 ± 0.23 | 2.37 ± 0.89 | 2.20 ± 0.50 | 5.01 ± 1.73 | 5.87 ± 0.04 | 10.40 ± 0.75 | 9.91 ± 0.44 |
| C 15:0 | 0.34 ± 0.02 | 1.53 ± 0.29 | 1.90 ± 0.04 | 0.47 ± 0.05 | 0.55 ± 0.02 | 0.47 ± 0.15 | 0.59 ± 0.01 | 0.98 ± 0.10 | 1.18 ± 0.04 |
| C 16:0 | 21.21 ± 0.94 | 23.79 ± 5.44 | 27.38 ± 0.11 | 19.07 ± 2.82 | 18.96 ± 1.36 | 15.02 ± 4.52 | 17.67 ± 0.28 | 27.6 ± 0.1 | 29.04 ± 0.39 |
| C 16:1ω9 | 0.37 ± 0.02 | 2.04 ± 0.30 | 2.39 ± 0.03 | 0.41 ± 0.23 | 0.40 ± 0.24 | 0.5 ± 0.18 | 0.41 ± 0.13 | 1.21 ± 0.28 | 1.37 ± 0.01 |
| C 16:1ω7 | 7.50 ± 0.32 | 19.85 ± 5.44 | 18.34 ± 0.36 | 10.2 ± 6.64 | 7.08 ± 1.79 | 13.65 ± 4.36 | 14.03 ± 0.33 | 29.4 ± 0.77 | 24.12 ± 1.16 |
| C 17:0 | 0.47 ± 0.02 | 0.68 ± 0.18 | 0.90 ± 0.03 | 0.61 ± 0.28 | 0.75 ± 0.12 | 0.54 ± 0.17 | 0.64 ± 0.01 | 0.62 ± 0.01 | 0.95 ± 0.04 |
| C DMA 18:0 | 0.60 ± 0.02 | 1.00 ± 0.19 | 1.10 ± 0.05 | 6.10 ± 0.58 | 6.15 ± 0.15 | 3.97 ± 0.87 | 4.76 ± 0.36 | 1.40 ± 0.05 | 1.34 ± 0.13 |
| C 18:0 | 6.00 ± 0.21 | 1.84 ± 0.22 | 5.12 ± 1.11 | 3.28 ± 0.98 | 3.84 ± 0.44 | 3.32 ± 0.93 | 3.95 ± 0.02 | 2.41 ± 0.23 | 4.78 ± 0.77 |
| C 18:1ω9 | 24.38 ± 1.01 | 13.16 ± 2.23 | 12.99 ± 1.28 | 1.84 ± 1.22 | 1.71 ± 0.27 | 1.14 ± 0.29 | 3.03 ± 0.25 | 7.20 ± 0.66 | 7.28 ± 0.23 |
| C 18:1ω7 | 2.80 ± 0.17 | 3.62 ± 0.84 | 3.70 ± 0.31 | 2.58 ± 0.49 | 2.33 ± 0.21 | 2.19 ± 0.65 | 2.30 ± 0.07 | 1.95 ± 0.28 | 1.75 ± 0.20 |
| C 18:2ω6 | 15.35 ± 0.62 | 2.64 ± 0.49 | 3.61 ± 0.07 | 0.64 ± 0.04 | 1.07 ± 0.05 | 0.86 ± 0.01 | 1.52 ± 0.12 | 1.59 ± 0.03 | 2.23 ± 0.30 |
| C 18:3ω6 | - | 0.03 ± 0.06 | - | 1.31 ± 0.56 | 1.05 ± 0.13 | 0.42 ± 0.1 | 0.48 ± 0.07 | 0.12 ± 0.01 | 0.08 ± 0.02 |
| C 18:3ω3 | 1.39 ± 0.04 | 0.71 ± 0.17 | 1.04 ± 0.06 | 0.58 ± 0.07 | 0.58 ± 0.02 | 0.69 ± 0.24 | 0.92 ± 0.03 | 0.31 ± 0.03 | 0.36 ± 0.02 |
| C 18:4ω3 | 0.83 ± 0.03 | 1.42 ± 0.26 | 1.64 ± 0.14 | 1.81 ± 0.93 | 1.74 ± 0.48 | 2.75 ± 0.88 | 3.35 ± 0.08 | 1.34 ± 0.04 | 1.20 ± 0.17 |
| C 20:1ω11 | 0.18 ± 0.00 | 0.17 ± 0.04 | 0.06 ± 0.11 | 1.02 ± 0.24 | 1.13 ± 0.09 | 0.70 ± 0.26 | 0.89 ± 0.03 | 0.4 ± 0.06 | 0.41 ± 0.08 |
| C 20:1ω9 | 0.64 ± 0.03 | 1.82 ± 0.62 | 3.63 ± 0.18 | 2.30 ± 0.63 | 3.03 ± 0.45 | 1.36 ± 0.39 | 2.21 ± 0.02 | 0.46 ± 0.09 | 0.67 ± 0.21 |
| C 20:1ω7 | 0.14 ± 0.00 | 0.03 ± 0.06 | - | 1.29 ± 0.28 | 1.19 ± 0.05 | 1.26 ± 0.5 | 1.33 ± 0.05 | 0.44 ± 0.09 | 0.37 ± 0.11 |
| C 20:2NMI1 | 0.14 ± 0.00 | - | 0.16 ± 0.02 | 1.75 ± 0.5 | 2.04 ± 0.35 | 1.74 ± 0.52 | 2.25 ± 0.31 | 0.17 ± 0.01 | 0.43 ± 0.13 |
| C 20:2NMI2 | - | - | - | 0.48 ± 0.11 | 0.47 ± 0.17 | 0.38 ± 0.2 | 0.42 ± 0.02 | 0.09 ± 0.02 | 0.1 ± 0.03 |
| C 20:2ω6 | 0.22 ± 0.03 | 0.12 ± 0.04 | 0.08 ± 0.07 | 0.50 ± 0.15 | 0.58 ± 0.04 | 0.43 ± 0.14 | 0.55 ± 0.04 | 0.1 ± 0.01 | 0.16 ± 0.03 |
| C 20:4ω6 | 0.96 ± 0.03 | 11.7 ± 19.4 | 0.53 ± 0.11 | 1.40 ± 0.41 | 1.83 ± 0.28 | 0.91 ± 0.22 | 1.12 ± 0.03 | 0.93 ± 0.11 | 1.12 ± 0.21 |
| C 20:4ω3 | 0.39 ± 0.03 | 0.22 ± 0.07 | 0.31 ± 0.07 | 0.45 ± 0.18 | 0.45 ± 0.07 | 0.51 ± 0.14 | 0.51 ± 0.03 | 0.21 ± 0.04 | 0.14 ± 0.03 |
| C 20:5ω3 (EPA) | 5.57 ± 4.08 | 2.13 ± 0.53 | 2.27 ± 0.01 | 24.38 ± 4.07 | 24.66 ± 1.25 | 18.62 ± 5.7 | 20.54 ± 0.35 | 5.74 ± 0.38 | 6.55 ± 1.00 |
| C 22:0 | 0.06 ± 0.00 | 0.80 ± 0.11 | 0.75 ± 0.10 | 0.02 ± 0.04 | 0.03 ± 0.05 | 0.14 ± 0.04 | 0.21 ± 0.02 | 0.63 ± 0.09 | 0.52 ± 0.20 |
| C 22:2NMID1 | - | - | - | 0.31 ± 0.07 | 0.47 ± 0.02 | 0.21 ± 0.06 | 0.37 ± 0.01 | 0.21 ± 0.03 | 0.2 ± 0.05 |
| C 22:2NMID2 | - | 0.07 ± 0.06 | 0.12 ± 0.01 | 1.92 ± 0.42 | 1.86 ± 0.09 | 1.49 ± 0.45 | 1.48 ± 0.08 | 0.66 ± 0.10 | 0.53 ± 0.10 |
| C 22:3 NMIT | - | - | - | 0.36 ± 0.04 | 0.37 ± 0.03 | 0.24 ± 0.08 | 0.25 ± 0.05 | 0.09 ± 0.01 | 0.09 ± 0.03 |
| C 22:4ω6 | 0.64 ± 0.05 | 0.33 ± 0.04 | 0.46 ± 0.10 | 1.25 ± 0.05 | 1.44 ± 0.02 | 1.01 ± 0.29 | 1.22 ± 0.03 | 0.44 ± 0.03 | 0.54 ± 0.06 |
| C 22:5ω6 | 0.37 ± 0.01 | - | 0.10 ± 0.10 | 0.2 ± 0.05 | 0.23 ± 0.02 | 0.16 ± 0.03 | 0.16 ± 0.01 | 0.06 ± 0.05 | 0.12 ± 0.01 |
| C 22:5ω3 | 1.16 ± 0.04 | 0.12 ± 0.13 | 0.21 ± 0.09 | 1.16 ± 0.25 | 1.35 ± 0.17 | 0.6 ± 0.14 | 0.75 ± 0.07 | 0.29 ± 0.02 | 0.37 ± 0.08 |
| C 22:6ω3 (DHA) | 4.08 ± 0.25 | 1.02 ± 0.2 | 0.95 ± 0.16 | 9.95 ± 4.24 | 10.47 ± 1.83 | 5.56 ± 1.55 | 6.21 ± 0.13 | 2.56 ± 0.21 | 2.11 ± 0.41 |
| Σ SAFA | 32.19 ± 1.37 | 37.69 ± 8.80 | 46.19 ± 1.27 | 25.82 ± 2.54 | 26.33 ± 1.27 | 24.49 ± 7.41 | 28.94 ± 0.30 | 42.63 ± 0.65 | 46.37 ± 1.18 |
| Σ MUFA | 36.04 ± 1.58 | 40.72 ± 8.58 | 41.14 ± 1.26 | 19.62 ± 6.64 | 16.86 ± 1.33 | 20.80 ± 6.33 | 24.19 ± 0.53 | 41.06 ± 0.43 | 35.97 ± 0.62 |
| Σ PUFA | 31.16 ± 2.97 | 10.44 ± 0.64 | 11.55 ± 0.24 | 48.44 ± 7.93 | 50.65 ± 2.46 | 50.73 ± 14.45 | 42.10 ± 0.30 | 14.90 ± 0.83 | 16.31 ± 1.73 |
| Σ DMA | 0.61 ± 0.02 | 1.00 ± 0.19 | 1.10 ± 0.05 | 6.10 ± 0.58 | 6.14 ± 0.14 | 3.96 ± 0.86 | 4.76 ± 0.35 | 1.39 ± 0.05 | 1.33 ± 0.12 |
| Σ PUFA ω3 | 13.45 ± 3.67 | 5.64 ± 1.27 | 6.45 ± 0.25 | 38.32 ± 7.42 | 39.25 ± 2.68 | 28.72 ± 8.47 | 32.27 ± 0.62 | 10.45 ± 0.61 | 10.72 ± 1.63 |
| ω3/ω6 | 0.77 ± 0.23 | 1.10 ± 0.88 | 1.34 ± 0.07 | 7.28 ± 0.35 | 6.33 ± 0.13 | 5.45 ± 4.37 | 6.38 ± 0.11 | 3.23 ± 0.18 | 2.53 ± 0.47 |
| Σ NMI | 0.14 ± 0 | 0.07 ± 0.06 | 0.28 ± 0.03 | 4.81 ± 0.87 | 5.20 ± 0.62 | 4.05 ± 1.26 | 4.77 ± 0.42 | 1.21 ± 0.16 | 1.33 ± 0.32 |
| Σ NMID | 0.14 ± 0 | 0.07 ± 0.06 | 0.28 ± 0.03 | 4.45 ± 0.85 | 4.83 ± 0.64 | 3.82 ± 1.18 | 4.51 ± 0.37 | 1.13 ± 0.15 | 1.25 ± 0.30 |
| Σ NMIT | - | - | - | 0.35 ± 0.04 | 0.37 ± 0.02 | 0.23 ± 0.07 | 0.253 ± 0.04 | 0.08 ± 0.00 | 0.08 ± 0.02 |

Table 3. Main fatty acid (FA) and FA classes of the salmon feed, seston and the mantle tissue, mussel digestive gland and feces of *M. edulis* measured at the monoculture and the IMTA site. The content is expressed as relative percentage of total FA (mean ± SD). The – indicates the FA was below the detection limit. SAFA = saturated FA, MUFA = monounsaturated FA, PUFA = polyunsaturated FA, DMA= dimethyl acetals FA, PUFA ratio for the ω3 and ω6 series, NMI= non-methylene-interrupted FA, NMID= NMI dienoic FA, NMIT= NMI trienoic FA.

| Sites | Biochemical composition | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Proteins (J mg$^{-1}$) | | | Carbohydrates (J mg$^{-1}$) | | | Lipids (J mg$^{-1}$) | | |
| | Mantle | Digestive gland | Feces | Mantle | Digestive gland | Feces | Mantle | Digestive gland | Feces |
| Monoculture | $9.36 \pm 0.45$ | $10.03 \pm 0.49$ | $7.26 \pm 0.21$ | $2.38 \pm 0.35$ | $2.30 \pm 0.13$ | $3.04 \pm 0.06$ | $4.79 \pm 1.26$ | $6.59 \pm 0.26$ | $6.18 \pm 0.61$ |
| IMTA | $9.07 \pm 0.39$ | $10.08 \pm 0.91$ | $8.21 \pm 0.62$* | $2.94 \pm 0.45$ | $2.27 \pm 0.04$ | $3.55 \pm 0.24$* | $4.27 \pm 0.60$ | $6.89 \pm 0.27$ | $7.00 \pm 0.41$* |

| | Ecophysiological measurements | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CI (%) | CR (l h$^{-1}$) | OIR (mg h$^{-1}$) | AR (mg h$^{-1}$) | VO$_2$ (ml h$^{-1}$) | VNH$_4$-N (µg h$^{-1}$) | O:N | SFG (J h$^{-1}$) | K$_2$ |
| Monoculture | $16.02 \pm 2.95$ | $4.16 \pm 1.27$ | $3.14 \pm 0.96$ | $2.21 \pm 0.60$** | $0.54 \pm 0.13$ | $44.76 \pm 18.14$* | $19.82$ | $38.71 \pm 11.73$** | $0.72 \pm 0.10$* |
| IMTA | $21.00 \pm 2.02$*** | $5.31 \pm 1.65$** | $2.88 \pm 0.89$ | $1.56 \pm 0.31$ | $0.58 \pm 0.09$ | $33.98 \pm 12.96$ | $24.36$ | $28.41 \pm 9.72$ | $0.64 \pm 0.13$ |

Table 4. Results on the biochemical composition (Joules per mg), condition index (CI) and ecophysiological rates integrating the Scope for Growth (SFG) of mussel *Mytilus edulis* measured at the monoculture and IMTA locations. The biochemical composition is referred to the mussels' organs (mantle tissue and digestive gland) and mussel feces. Statistical differences within the same column are indicated by *$P<0.05$, **$P<0.01$, ***$P<0.001$.

### 3.3. Mussel feces proximate and fatty acid composition

Mussels egested 0.33 and 0.37 mg DW $h^{-1}$ $ind^{-1}$ at the IMTA and monoculture locations. The OC, proteins, carbohydrates and lipids of the mussel feces were higher at the monoculture than at the IMTA site (Tukey HSD, $P<0.001$; Table 2; Fig. 2). However, mussel feces had lower energy content at the monoculture than at the IMTA station, as significant differences were detected for the protein ($F_{(1,4)}=13.06$, $P<0.05$), carbohydrate ($F_{(1,4)}=10.85$, $P<0.05$) and lipid ($F_{(1,4)}=8.20$, $P<0.05$) energetic content among sites (Table 4). Mussel feces FA signature was constituted mostly by 14:0, 16:0 and 16:1ω7, while SAFA were followed by MUFA and PUFA (Table 3). The NMDS plot showed a perfect spatial separation for that the FA profile of the mussel feces (Fig. 3d, stress 0.01, one-way ANOSIM, global $R$:1, $P=0.1$). The average dissimilarity between sites was 6.14 % and was explained by 18:0, fish feed FA biomarker 18:2ω6 and the MUFA 16:1ω7 (SIMPER, 16.1, 6.6 and 5.8 %) (Fig. 4d). The feces of mussels at the IMTA site had a higher content of SAFA, 18:0 ($F_{(1,4)}=26.09$, $P<0.01$), fish feed biomarker 18:2ω6 ($F_{(1,4)}=13.50$, $P<0.05$; Fig. 5a) and 20:2NMI 1 ($F_{(1,4)}=10.89$, $P<0.05$). The opposite trend was observed for 16:1ω7 ($F_{(1,4)}=43.23$, $P<0.01$). Feces contained elevated amounts of diatom biomarkers, while dinoflagellate and bacteria-specific FA biomarkers were present to a lesser extent (Fig. 5b).

### 3.4. Fish feed composition and incorporation in tissues

Fish feed contained a high OC and energy content, while proteins were higher than lipids and carbohydrates (Fig. 2). Fish feed further showed a very high relative content of biomarkers 18:1ω9 and 18:2ω6, balanced proportions of SAFA, MUFA and PUFA and a typically low ω3/ω6 ratio (Table 3). The FA profile of the fish feed segregated perfectly from the rest of the samples collected at IMTA station (Fig. 3e, stress 0.04, gobal $R$:1, $P=0.1$). The overall NMDS plot showed three separated groups: the fish feed, the seston-feces and mussels' organs. Seston from the IMTA site and fish feed differed in the contents of 18:2ω6, 20:1ω9 and 22:6ω3

(SIMPER, average dissimilarity 22.2 %). Fish feed and the mantle tissue differed in the amounts of 18:1ω9 and 18:2ω6, which were more abundant in the feed, while EPA was higher in the mantle (SIMPER, average dissimilarity 30.88 %). Fish feed and the gland differed in the same FA (SIMPER, 27.34 %). Lastly, fish feed and mussel feces dissimilarity was explained by 18:2ω6, 18:1ω9 and 16:1ω7 (SIMPER, 22.24 %). Overall, the mussel mantle and the digestive gland had quite a similar FA profile (SIMPER, average dissimilarity 11.3 %) and differences were attributed to 14:0, 16:1ω7 and 22:6ω3. On the other hand, seston and mussel feces grouped together (SIMPER, average dissimilarity 16.2 %) and differed in the amounts of 20:1ω9, 20:5ω3 and 20:4ω6.
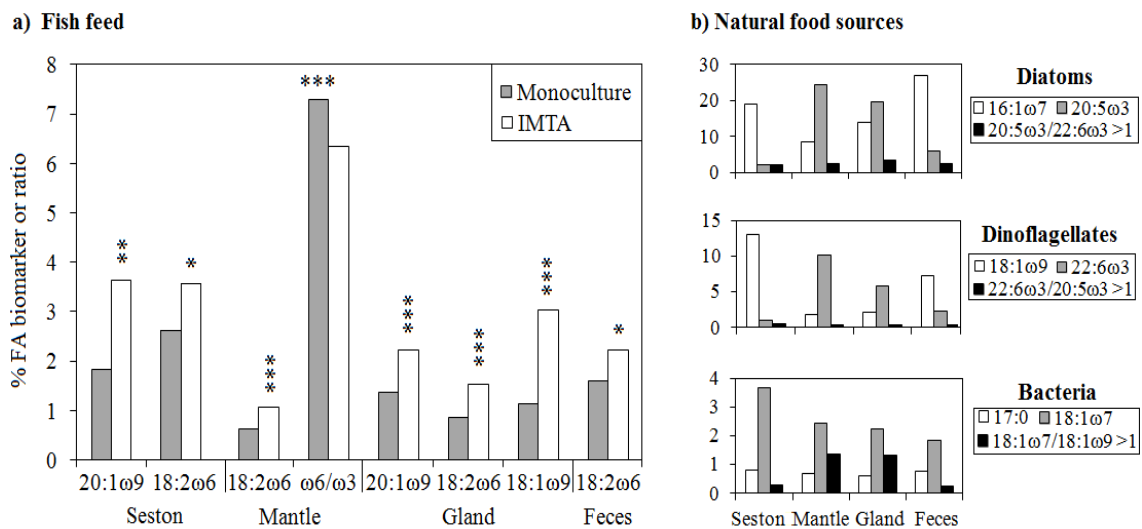


Fig. 5. Differences in fatty acid (FA) composition for: a) the main fish feed biomarkers found between seston, mussel organs and feces at the monoculture and the IMTA site and, b) main diatom, dinoflagellates and bacteria biomarker percentages and ratios found in the seston and mussel samples. Significant differences between sites are denoted by *P<0.05, **P<0.01 and ***P<0.001.

### 3.5. Stable Isotopes of food sources and mussel feces

The salmon diet signatures had average values of -19.9 ± 0.3 ‰ $\delta^{13}C$ and 4.2 ± 0.1 ‰ $\delta^{15}N$. The

$\delta^{13}C$ and $\delta^{15}N$ signatures in the seston differed between sites, as there was an enrichment of $\delta^{13}C$

and $\delta^{15}N$ at the monoculture site (Fig. 6). There appeared to be some fractionation or trophic

shift occurring in mussels' fecal pellets, but the same significant trends of $\delta^{13}C$ and $\delta^{15}N$

enrichment were apparent for the monoculture site (ANOVA, $P<0.05$; Fig. 6).
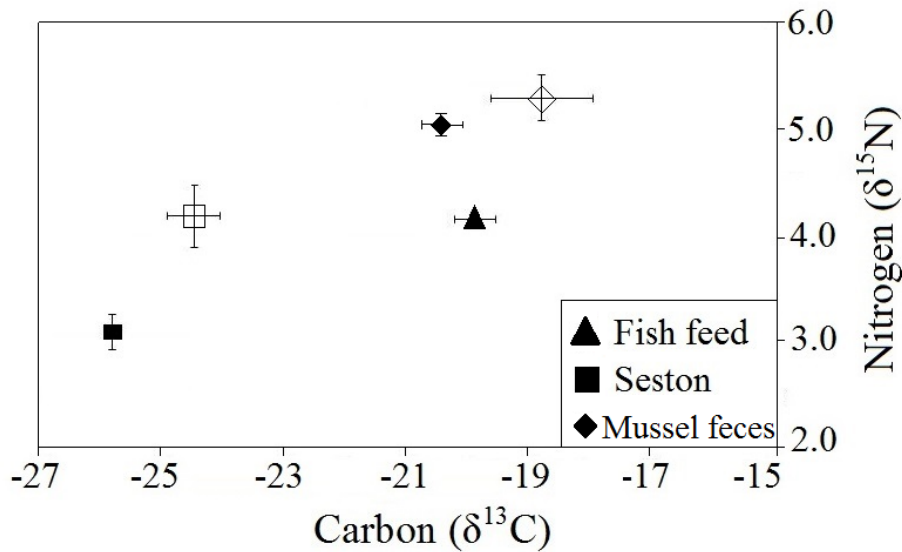


Fig. 6. Bivariate plot of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) of the potential food sources (salmon

feed and seston) and resulting mussel feces from the IMTA site (filled symbols) and the

monoculture site (open symbols). Error bars represent the standard error of the mean.

### 3.6. Physiological rates

One-way ANOVA followed by post-hoc testing indicated that the clearance rate (CR) of

*Mytilus edulis* was significantly greater at the IMTA site, while the absorption rate (AR) and the

ammonia excretion rate (VNH$_4$-N) were greater for monoculture mussels (Tables 4 and 5). The

results indicated that the Scope for Growth (SFG) and the growth efficiency (K$_2$) of IMTA

mussels was 26.60 % and 11.11 % lower, respectively, than that of mussels from the monoculture site (Tables 4 and 5).

## 3.7. Condition index (CI)

The CI of mussels at the monoculture site was determined on mussels with $56.95 \pm 1.53$ mm shell length, $1.00 \pm 0.25$ g of tissue DW and $6.39 \pm 0.99$ g of shell DW (Table 4). The CI of IMTA mussels was determined on individuals with $52.04 \pm 1.33$ mm shell length, $0.65 \pm 0.06$ g of tissue DW and $3.12 \pm 0.44$ g of shell DW (Table 4). The CI was significantly higher for IMTA mussels than monoculture individuals (ANOVA, $P<0.001$; Table 5).

# 4. Discussion

This study demonstrated that mussels cultured under open-water IMTA conditions were assimilating and egesting fish feed FA biomarkers, although this dietary enhancement was within the range of natural variations of seston loads and was not enough to increase the Scope for Growth and energy reserves compared with monoculture mussels.

## 4.1. Dietary enhancement of natural food supply by fish feed particulate waste

In coastal ecosystems, seston nutritional quality is highly variable, partially depending on the composition and biomass of the phytoplankton. High protein, lipid and PUFA contents are indicators of good dietary quality for bivalves (Navarro and Thompson, 1995; Ríos et al., 1998; Budge et al., 2001; Alkanani et al., 2007; Isla et al., 2010; 2012). Our results showed significantly higher levels of chlorophyll at the IMTA farm relative to the monoculture site, although these levels were within the wide range reported for the Bay of Fundy (MacDonald et al., 2011; Lander et al., 2013).

| Effect | df | SS | MS | *F*-value | *P*-value |
|---|---|---|---|---|---|
| *Clearance rate* | | | | | |
| Site | 1 | 20 | 20 | 9 | <0.01** |
| Error | 59 | 130 | 2 | | |
| | | | | | |
| *Organic ingestion rate* | | | | | |
| Site | 1 | 0.97 | 0.97 | 1.13 | 0.29 ns |
| Error | 59 | 50.96 | 0.86 | | |
| | | | | | |
| *Absorption rate* | | | | | |
| Site | 1 | 1.87 | 1.87 | 8.06 | <0.01** |
| Error | 16 | 3.71 | 0.23 | | |
| | | | | | |
| *Respiration rate* | | | | | |
| Site | 1 | 0 | 0 | 0 | 0.96 ns |
| Error | 42 | 1 | 0 | | |
| | | | | | |
| *Ammonia excretion rate* | | | | | |
| Site | 1 | 1266.13 | 1266.13 | 5.25 | <0.05* |
| Error | 42 | 10123.79 | 241.04 | | |
| | | | | | |
| *O:N ratio* | | | | | |
| Site | 1 | 224.9 | 224.9 | 2 | 0.17 ns |
| Error | 42 | 5038.51 | 119.96 | | |
| | | | | | |
| *Scope for Growth* | | | | | |
| Site | 1 | 1392.18 | 1392.18 | 10.2 | <0.01** |
| Error | 53 | 7236.58 | 136.54 | | |
| | | | | | |
| *Net growth efficiency* | | | | | |
| Site | 1 | 0.03 | 0.03 | 4.95 | <0.05* |
| Error | 53 | 0.33 | 0.01 | | |
| | | | | | |
| *Condition index* | | | | | |
| Site | 1 | 272.80 | 272.80 | 43.38 | <0.001*** |
| Error | 43 | 264.09 | 6.29 | | |

Table 5. Results of one-way ANOVA testing the influence of the site on the physiological rates and Condition index obtained in the Bay of Fundy. Significant differences are denoted by *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ns (not significant).

Chlorophyll at the IMTA farm was recorded coincident with a period of sediment resuspension, as demonstrated by the comparatively lower quality (*f*), energetic content, proteins, carbohydrates and lipids suspended in the seston relative to the monoculture site. We also recorded elevated concentrations of TPM and dilution of the organic matter by PIM in a simultaneous study (Irisarri et al., 2013). Considering the high winds during the study period (NW wind of 35-39 km h$^{-1}$) and that the IMTA farm was moored in shallower waters than the monoculture, it is not surprising that there was higher turbidity at the IMTA site. The increased turbidity could have masked any organic input coming from the fish cages, even if we took seston samples directly at the cages during the morning salmon feeding session. Previous studies detected increments in fish POM at 0 and 5 m from the fish cages through time-series measurements (Lander et al., 2013). Similarly, Modica et al. (2006) and Sarà et al. (2009) measured higher accumulations of carbohydrates and proteins in seston near fish net-pens than at a control sites. Given that our sampling was a single daily measurement of chlorophyll and seston loads at each location, and that the Bay of Fundy is a macro-tidal ecosystem with marked shifts in seston quantity and quality over small spatial scales (MacDonald et al., 2011; Nelson et al., 2012), we cannot conclude that chlorophyll levels were constantly enhanced at the IMTA site. In fact, more intensive sampling series in the Bay of Fundy found no consistent enhancement of chl-*a* at salmon cages, probably because phytoplankton and nutrients were effectively dispersed (MacDonald et al., 2011; Lander et al., 2013). Thus, our data should be considered as a mere snapshot of the dietary environment. Accordingly, more intensive sampling series are still required to further assess if chlorophyll levels and particulate organic matter are enhanced at this particular IMTA site. Still, considering that the energetic tides at the IMTA site effectively transport ammonia, nitrate and nitrite away from the cages (Robinson et al., 2005), any additional chlorophyll production might not be available for mussels consumption at all times.

Even if we did not detect a dietary enhancement through short-term bulk seston measurements, our results demonstrated that seston at the IMTA site significantly incorporated fish feed FA biomarkers 20:1ω9 and 18:2ω6. The higher levels of 20:4ω6 in seston at the monoculture site

suggested that arachidonic acid was probably not an adequate fish feed FA biomarker in this study. This FA could indicate the presence of detrital or macroalgal material in the seston (Ventrella et al., 2008; Richoux et al., 2014). The FA profile of the seston was similar to the plankton profile described in Newfoundland (Canada) during the spring bloom (Parrish et al., 2005) and over a two year period (Budge et al., 2001; Alkanani et al., 2007), although PUFA, EPA and DHA in the latter studies showed greater proportions. In this study, the higher contribution of biomarkers EPA, $16:1\omega7$ and the ratio EPA/DHA>1 could be linked to a predominance of diatoms over dinoflagellates and bacteria in the phytoplankton community during July. The stable isotope (SI) analysis of the seston was consistent with those from the FA in discriminating between the two study locations. This separation suggests that SI signatures are also a good technique for the comparative assessment of environmental conditions and could be used as part of a larger evaluation package in ecological studies. Using SI could give an independent estimate of the dietary proportions from different sources (Garton et al., 2005; Gao et al., 2006; Atkinson et al., 2010; Slater and Carton, 2010).

### 4.2. Physiological measurements

The present research revealed significant physiological differences for *M. edulis*, since IMTA mussels showed comparatively higher clearance rate (CR) and lower absorption efficiency (AE), absorption rate (AR), ammonia excretion ($VNH_4$-N), Scope for Growth (SFG) and net growth efficiency ($K_2$) than monoculture individuals. The CR of bivalves is subjected to physiological regulation depending upon short-term and seasonal variations in the quality and concentration of seston. Generally, the CR increases with seston quality (chl-*a*, percent POM) (MacDonald and Ward, 1994; Okumus and Stirling, 1994; Hawkins et al., 1999) and remains constant up to a threshold concentration of TPM and chl-*a*, above which the CR decreases (Bayne and Widdows, 1978; Widdows et al., 1979; Hawkins et al., 1999; Riisgård, 2001; Velasco and Navarro, 2005; Filgueira et al., 2009). The CR of IMTA mussels probably increased with the significantly higher chlorophyll and TPM levels, as seston loads did not

surpass the upper physiological threshold above which the CR declines. Concurrently, a decline in CR was not observed for bivalves feeding in low turbidity ecosystems (Okumus and Stirling, 1994; Babarro et al., 2000b; MacDonald and Ward, 1994) and reductions in the valve aperture and water flow through the gills were only described for very turbid environments (>200 mg l$^{-1}$ TPM) (Bayne and Widdows, 1978; Widdows et al., 1979; Velasco and Navarro, 2005). Similarly, an upper threshold for the CR was found above 1.1 and 26.91 µg chl-$a$ l$^{-1}$, although this concentration varies among bivalve species and depends on the natural levels occurring at each ecosystem (Hawkins et al., 1999; Filgueira et al., 2009, respectively).

Despite the high chlorophyll levels, the poorer organic fraction of the seston ($f$) resulted in a lower AE (Irisarri et al., 2013) and AR for IMTA mussels. The decline in AE was accompanied by the egestion of feces with higher energetic content than the diet. This finding might be explained by: a) reduction in the residence time of the food through the gut due to higher CR and a limited intestinal capacity; b) larger percentages of non-absorbed food being lost through the fecal pellets; and c) an increased production of metabolic fecal losses (MFL) that enriched the energetic content of the feces. MFL are endogenous components –mainly mucus, epithelial cells abraded from the digestive tract, extracellular enzymes and intestinal bacteria– which are not reabsorbed during digestion and can represent an important energetic loss for bivalves (Hawkins et al., 1990; Navarro and Iglesias, 1993). The significantly lower OC and biochemical substrates found for mussels' feces at the IMTA site reflected the comparatively poorer quality of the seston. Similarly, variations in the OC of the diet were quickly reflected on the OC of *Mytilus* spp. feces feeding on algae, fish feed 'fines' or feces under simulated IMTA conditions (Liutkus et al., 2012). Thus, it appears that feces composition can vary rapidly in dynamic feeding environments.

In this study, the lower metabolic expenditure of IMTA mussels probably had an irrelevant influence on the SFG, as costs associated with ammonia excretion in mussels are considered negligible (Widdows and Staff, 2006) and represented only 6-9% of the metabolic loses in this study. On the other hand, the higher VNH$_4$-N and lower O:N ratio of monoculture mussels

appeared to be associated with differences in spawning timing among both mussel populations. This explanation is consistent with the lower condition index recorded for monoculture specimens discussed below. An increase in excretion and reductions in O:N index could be linked with a higher protein catabolism after gamete maturation (Babarro et al., 2000a). This pattern has been previously described for post-spawning bivalves (Worrall et al., 1983; Barber and Blake, 1985).

The SFG is the energy available for growth and reproduction from the ingested food after energy losses from respiration and excretion (Albentosa et al., 2012). Based on the enhanced CR, similar $VO_2$ and reduced $VNH_4$-N of co-cultured mussels, we proposed that the lower SFG and $K_2$ of IMTA mussels were mainly attributable to the reduction in food absorption under a significantly lower nutritional diet. Navarro et al. (1991) reported higher CR and AE for *M. galloprovincialis* cultured on rafts were seston had higher POM. The authors found that improved CR and AE were the main physiological rates accounting for an enhanced SFG. Laboratory measurements using prepared diets also found that higher CR and AE resulted in an enhanced SFG (Labarta et al., 1997). Previous studies have also estimated a significant positive correlation between the SFG of *M. galloprovincialis* with AE (Albentosa et al., 2012), the SFG of *M. edulis* with POM (Okumus and Stirling, 1994) and the SFG of *Perna viridis* with *f* (Wong and Cheung, 2003).

### *4.3. Assimilation and egestion of fish feed particulate waste*

Shellfish proximate composition fell within the range of values reported for the mantle and gland of natural populations of mussels (Zwaan & Zandee, 1972; Zandee et al., 1980; Martínez-Pita et al., 2012). Our results agreed with previous studies that measured none or very slight increases in proteins, carbohydrate and glycogen content in *M. edulis* reared close to salmon farms (Taylor et al., 1992; Cheshuk et al., 2003). Conversely, laboratory experiments found increased lipid and protein content for *M. edulis* feeding on salmon pellets (Redmond et al.,

2010; Both et al., 2011; 2012), although these results are not directly comparable as fish effluents are less dispersed in the laboratory. The equivalent proximate composition of mussel organs demonstrated that, on the long-term, mussels were feeding on seston of similar characteristics and resuspension events at the IMTA site were probably a transitory event.

As in the case of the seston samples, the analysis of the FA profile of mussel tissues revealed that bivalves were able to assimilate fish feed FA biomarkers (20:1ω9, 18:2ω6 and 18:1ω9) and thus, utilize some of the particulate feed waste as part of their diet. IMTA mussels also displayed a lower ω3/ω6 ratio in their mantle tissue; a characteristic that has been associated with the assimilation of fish feed particles by mussels (Redmond et al., 2010). In addition, the FA signature of mussel tissues and feces reflected the flow of diatom-derived FA biomarkers from seston up to higher trophic levels, while the low percentage of dinoflagellate and bacteria FA biomarkers suggested that diatoms were the major natural food source for the filter-feeders during this study. The absorption of feed particulate waste was further reflected through the significantly higher levels of 18:2ω6 contained in the egested fecal pellets. This biomarker was very abundant in the salmon feed and seston from the IMTA site, suggesting that mussels acquired this FA through feeding on the suspended fish feed waste. High proportions of fish feed biomarkers 20:1ω9, 18:2ω6, 18:1ω9 and low ω3/ω6 ratio were also found in mussels cultured close to fish pens or supplemented with crushed fish pellets or fish effluents (Gao et al., 2006; Redmond et al., 2010; Both et al., 2011; 2012; 2013; Handå et al. 2012 a).

It is important to highlight that IMTA mussels were highly energetic and contained elevated levels of ω3 PUFA EPA and DHA, low levels of ω6 FA and high ω3/ω6 ratio, which have been related with the prevention of cardiovascular and cancer diseases (Grienke et al., 2014). A similar nutritional quality has been reported for edible mussels *Mytilus galloprovincialis* (Fernández-Reiriz et al., 1996; Freites et al., 2002 a; b; Fuentes et al., 2009) and *M. edulis* cultured in suspension (Budge et al., 2001; Alkanani et al., 2007). The absence of 20:2NMI2, 22:2NMID1 and 22:3NMIT in the seston suggested that *M. edulis* may have synthesized these FA from their precursors (Zhukova, 1991; Garrido and Medina, 2002; Alkanani et al., 2007; Ventrella et al., 2013) in addition or in replacement of ω3 and ω6 PUFA (Barnathan, 2009).

This can represent an adaptive strategy for mussels, which have a limited ability to synthesize EPA and ARA from precursors 18:2ω6 and 18:3ω3, owing to the lack of Δ6 desaturase (Ventrella et al., 2013). Overall, the integrated results of the physiological energetics, the PA and FA analyses pointed that IMTA mussels mostly fed on natural seston. Any additional energy input provided through uneaten feed particles, salmon feces, or increases in phytoplankton, did not appear to enhance the SFG and $K_2$ of mussels during short-term resuspension events. Given that this study was performed during the summer, a period of favourable conditions for mussel growth, it is probable that fish POM or fish-derived plankton might only meaningfully augment the SFG during extended periods of food scarcity in winter and autumn or perhaps when stocked at higher biomass densities (Cheshuk et al., 2003; Handå et al., 2012 a; Lander et al., 2013). Nonetheless, the presence of feed biomarkers in mussel feces suggested that the organic loading of the fish farm was being moderately biomitigated. Mussel feces were proven to have very high energetic and nutritional value, so deposit-feeders of great commercial interest like the sea cucumber could be cultured alongside the mussels to further exploit the bivalve feces. Mussel feces have a lower benthic impact relative to salmon feces, since slower settling speeds allows dispersion of bivalve biodeposits at greater distances (Liutkus et al., 2012). The recycling of uneaten feed particles by mussels, and the subsequent assimilation of mussel biodeposits into the benthic food web, might result in a lower benthonic organic enrichment compared with fish monoculture farms. Interestingly, the $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures from the feces were significantly different than the seston nourishing the mussels. It is known that there can be some fractionation of $\delta^{13}C$ and $\delta^{15}N$ isotopes during various cellular processes occurring with metabolism of ingested food items (Deudero et al., 2009), but that percentages change in fractionation, or trophic shift was higher than expected. While this may have been due to differential uptake in the gut of the lighter isotopes, it may also have been a result of the fecal matter representing a meal ingested prior to the onset of this experiment. Because of the relatively short duration of this work, it is not possible to determine the exact cause, but it may be worth considering in future research.

### 4.4. Condition index

The CI is generally used by mussel farmers to determine shellfish market quality (Orban et al., 2002; Albentosa et al., 2012). The results of the CI were within the range reported for *M. edulis* in Atlantic Canada (Filgueira et al., 2013). The lower CI at the monoculture site, along with the results on the ammonia excretion and O:N index, appeared to indicate variability in spawning timing among locations. Spawning of *M. edulis* in the Bay of Fundy typically starts in June and extends throughout summer (Lander et al., 2010; 2012). Mismatches on the CI and glycogen reserves between closely located mussel farms have been linked to differences in food availability, which are intimately coupled with the gametogenic cycle (Fernández-Reiriz et al., 1996; Lander et al., 2012). Our observations on IMTA mussels confirmed that gametes were starting to be expelled at the time of the experiment, whereas the results suggested that mussels from the monoculture site were recovering from a recent spawning event. Concurrently, Lander et al. (2012) reported peak CI at the beginning of spawning in June, while a loss in CI up to 40 % occurred throughout the spawning period in the Passamaquoddy region of the Bay of Fundy.

### 4.5. Conclusion

The assimilation and egestion of fish feed FA biomarkers confirmed that some feed waste was being incorporated and partially bio-mitigated by IMTA mussels. However, the comparable PA and the lower SFG measured for IMTA mussels indicated that feed waste constituted a small part of the mussels' diet and did not compensate for the temporary lower quality of the seston during resuspension events. These results suggest that uneaten feed particles may increase the SFG in IMTA systems experiencing food scarcity. We consider that a multi-indicator approach could provide a more holistic vision of the effectiveness and benefits of integrated fed-extractive IMTA aquaculture under different environmental conditions.

# Chapter 7

Temporal and spatial variations in proximate composition and Condition Index of mussels *Mytilus galloprovincialis* cultured in suspension in a shellfish farm

## ABSTRACT

We compared the seasonal variations in Condition Index (CI) and proximate composition of the mantle and the digestive gland of mussels (*Mytilus galloprovincialis*) cultivated at outer and inner regions of a raft polygon. The results are discussed in the context of the energy balance. The proximate composition and CI varied with the seasonal fluctuations in seston composition and the reproductive cycle described for the Galician Rías. Seston's nutritional quality peaked during the spring bloom and descended during winter downwelling. Proteins were first depleted in the gland during autumn, while the mantle maintained high levels until summer. Similarly, lipids were highest in the mantle during winter and decreased following the spring spawning, suggesting transference of reserves from the gland to the mantle to support gametogenesis. In contrast, glycogen was stored in the mantle during the summer and exhausted during winter, when food % POM was lowest. This opposite pattern suggested that glycogen was probably converted to lipids during gamete development. The variations in CI significantly correlated with the accumulation and expenditure of reserves. Mussels harvested in autumn had the highest CI and biochemical reserves, while minimum CI was in winter, when mussels had a low energy balance. Resuspension events in autumn-winter significantly diluted the particulate organic matter suspended at the innermost raft (38.91% POM) compared with the outer raft (60.52% POM). This was reflected in short-term reductions in CI, proteins' and lipids' reserves in innermost mussels. These temporal increases in turbidity did not seem to significantly affect bivalves' proximate composition and meat yield over a longer time scale.

**Key words:** *Mussel mantle; mussel digestive gland; proximate composition; Condition Index; spatio-temporal variations.*

# 1. Introduction

Bivalve mollusks like mussels, clams and oysters constitute highly nutritive seafood with an increasing demand and revenue in international markets (Orban et al., 2002; 2006; Fuentes et al., 2009; Karnjanapratum et al., 2013; Pogoda et al., 2013). Mussel *Mytilus galloprovincialis* is the most representative product coming from Spanish aquaculture, with 250,000 tons year$^{-1}$ which constitute 75 % of the total national aquaculture production (Labarta et al., 2004). Extensive mussel farming is mainly practiced in the Galician Rías (NW Spain), where mussels are cultured on ropes hanging from floating wooden structures known as rafts that are grouped in polygons. The phytoplankton blooms registered during the upwelling events in the Galician Rías from March to October support the third largest mussel production in the world (Figueiras et al., 2002). Mussels from the genus *Mytilus* are an important dietary source of proteins, ω3 polyunsaturated fatty acids (PUFA), glycogen and minerals (Fernández-Reiriz et al., 1996; Orban et al., 2002; Fuentes et al., 2009; Grienke et al., 2014). The majority of the mussels are consumed as fresh seafood (more than 50 % of the production), while the remaining production is processed as canned or frozen mussels (~35 and 15 %, respectively) (APROMAR, 2013). Mussels are sold at a competitive price relative to other bivalves, favouring both their domestic and international consumption.

The biochemical composition, together with the Condition Index (i.e. meat yield), are useful indicators of the nutritional and commercial quality of bivalves (Orban et al., 2002; 2006). These parameters fluctuate as a result of the synergistic interaction between the variations in the seston –the natural diet of suspension-feeders– quality and quantity and bivalves' reproductive cycle (Mathieu & Lubet, 1993; Fernández-Reiriz et al., 1996; Tavares et al., 1998; Pérez-Camacho et al., 1995; 2003; Orban et al., 2002; 2006; Freites et al., 2003; Park et al., 2011; Suárez et al., 2013; Baek et al., 2014). The reproductive cycle of *M. galloprovincialis* in the Galician Rías starts with the development and ripening of the gonad during autumn-winter. A major spawning event takes place during the spring upwelling season, after which the gonad restores and leads to secondary spawning events during late summer or early autumn (Villalba,

1995; Peteiro et al., 2011; Súarez et al., 2013). Suspension-feeders like *M. galloprovincialis* rely upon the seasonal and spatial variations of the suspended particulate matter that comprises the seston. In coastal areas with high primary production like the Galician Rías, seston nutritional value typically reaches highest levels during upwelling events (March-October) and lowest during downwelling episodes the rest of the year (Figueiras et al., 2002). In addition to seasonal variations, spatial changes in the feeding environment are common in coastal areas and can result from resuspension of bottom material due to storms or tidal cycles, changes in current speed or variable riverine nutrient outflow (Cranford et al., 2011). The resuspension of inorganic particles generally increases the total concentration of seston, but dilutes the amount of organic particles suspended in the water column, reducing the quality of the food available for bivalve filter-feeders (Cranford et al., 2011). The assessment of the proximate composition and Condition Index could help to indicate the nutritional value of bivalves feeding in a dynamic coastal environment. In this way, numerous studies have detected that the amount of biochemical reserves and the Condition Index can vary substantially among bivalves cultured in nearby sites within the same embayment (Fernández-Reiriz et al., 1996; Marin et al., 2003; Norkko and Thrush, 2006; Baek et al., 2014; Pérez-Camacho et al., 2014), or in proximate locations in a given coastal ecosystem (Okumus and Stirling, 1998; Kang et al., 2000; Sasikumar and Krishnakumar, 2011), owing to spatial differences in chlorophyll-*a*, food availability and quality.

This survey examined the influence of seasonal and geographical environmental changes in the gross biochemical composition and Condition Index of mussels (*Mytilus galloprovincialis*) cultured on a raft polygon in the Ría Ares-Betanzos, on the coast of Galicia (N.W. Spain). We compared the composition of the mantle and the digestive gland of bivalves cultured at two rafts, in the inner and outermost regions of the polygon, with the aim of investigating if shellfish composition varied with the tissue and the location of the culture units. The biochemical characteristics of the natural diet were monitored to understand the seasonal and spatial variations on the food available for the bivalves. This experiment was part of a broader study

that examined seasonal and spatial variations in the physiological energetics (Scope for Growth) and growth performance of *M. galloprovincialis*. Thus, the dynamics of the biochemical components and the CI are discussed in the context of changes in the energy balance and growth according to Irisarri et al. (2013; 2014 a, 2014 b).

## 2. Materials and methods

### 2.1. Study area and experimental design

The Ría Ares-Betanzos is a V-shaped coastal inlet with a total area of 272 km$^2$, a length of 19 km, a maximum width of 4.7 km at the mouth of the embayment and a water depth ranging from 2 to 43 m. The tide is semidiurnal and ranges from 0.02 to 4.14 m during neap and spring tides, respectively (Sánchez-Mata et al., 1999). The Ría receives an average freshwater discharge of 30 m$^3$ s$^{-1}$ from rivers Eume and Mandeo (Álvarez-Salgado et al., 2011). Lorbé and Arnela raft polygons (see Fig. 1) constitute the main mussel-farming regions of the Ría Ares-Betanzos, which has a total production of 10,000 tons mussel year$^{-1}$ (Labarta et al., 2004). This study was conducted on two mussel rafts from the Lorbé raft polygon, placed in the south-western coast of the Ría Ares-Betanzos, Spain (Fig. 1; 43°23'24.74''N, 8°17'48.30''W). The first raft was located on the innermost side of the polygon at 14 m depth and between 500-700 m N of the nearest coastline. The second raft was moored at the outer side of the polygon at 16 m depth and between 700-1000 m N of the coast. Rafts were sampled over the course of two consecutive days during the summer (July 2010), autumn (October 2010 and 2011 for any inter-annual variability), winter (February 2011) and spring (May 2011). In this way, we aimed to characterize the biochemical composition of mussels during the four main different hydrographical periods of the Galician Rías: 1) summer upwelling, 2) autumn bloom, 3) winter mixing and 4) spring bloom. The water in Lorbé raft polygon is well mixed and average current speeds documented at 1 m depth within the polygon average 1.7 cm s$^{-1}$, although maximum speeds can reach 15.5 cm s$^{-1}$ (Piedracoba et al., 2014). Sediments in Lorbé consist mainly of fine

sandy mud with a mean grain size of 0.01 to 0.15 µm in diameter, with 0-8 % gravel, 2-56 % sand, 0-36% silt and 7-18 % clay (Sánchez-Mata et al., 1999).
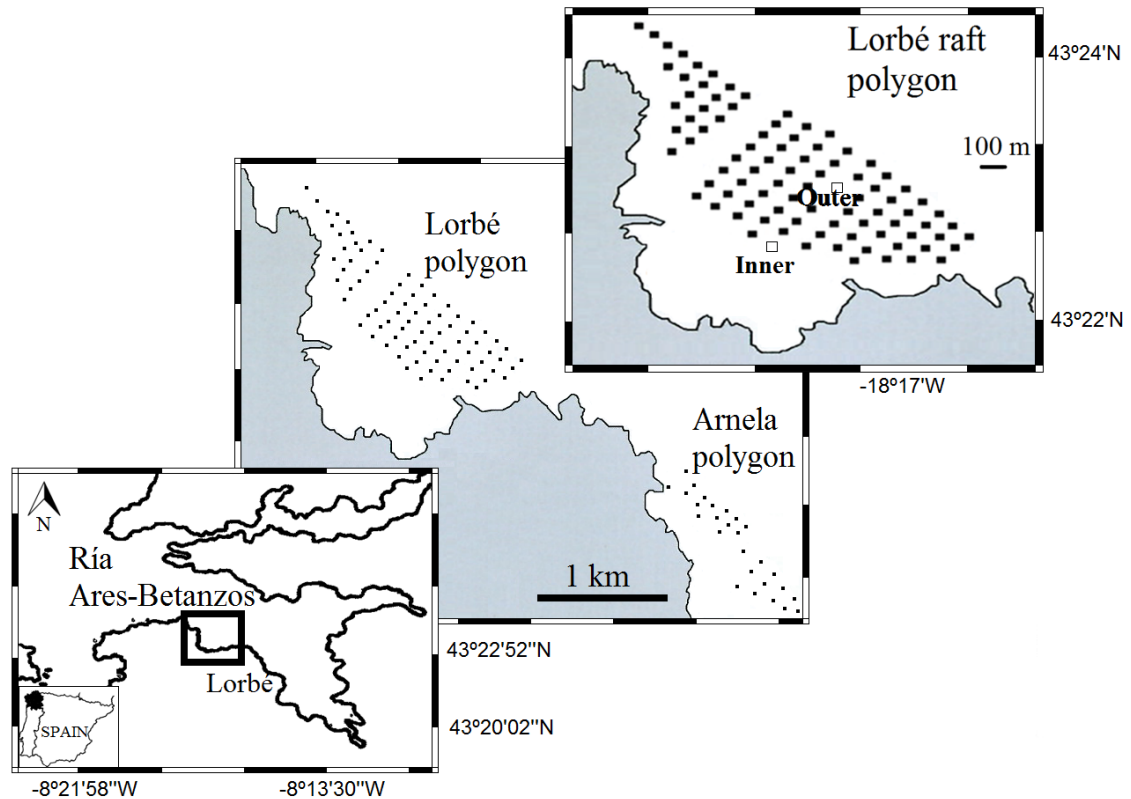


Fig. 1. Map of the study area showing the locations of the outer and inner rafts in the Lorbé mussel *Mytilus galloprovincialis* raft polygon (43°23'24.74''N, 8°17'48.30''W). The mussel polygon is in the Ría Ares-Betanzos, in Galicia, on the N.W. coast of Spain.

## 2.2. Environmental conditions

Physico-chemical parameters were monitored weekly during the months of July, October, February and May with a multiparameter probe YSI 556 to determine the temperature of the water (T, ºC), salinity (S, ‰), pH and oxygen ($O_2$, mg $l^{-1}$) at both rafts at a depth of 3 m.

During each monthly sampling, seawater was pumped from 3 m depth and sieved through a 50 µm mesh to eliminate large particles. Three replicates of 1 l of water were collected to characterize the particulate organic matter (%POM) and the gross biochemical composition of the seston. Water was filtered onto formiate-prewashed and precombusted (450 °C, 4 h)

Whatman GF/F filters. Filters were rinsed with isotonic ammonium formate (0.5 M) to remove salts, dried overnight and frozen at -50 °C until lyophilization. Filters for gross biochemical determinations were stored at -20 °C until further homogenization with a mortar and a pestle. Another series of seston filters were utilized for %POM determinations. These filters were dried at 110 °C for 12 h until constant weight, cooled in a desiccator and weighed with an accuracy of 0.001 mg using an electronic microbalance (Sartorius M3P, M3P-000V001). Samples were placed in pre-weighed aluminium pans and ashed at 450 ºC for 3 h, cooled in a desiccator and re-weighed. The difference between the dry weight and ashed weight indicated the % POM. Levels of chlorophyll-*a* were measured simultaneously in a parallel study (Irisarri et al., 2014 a).

## 2.3. Mussel sampling and biometric measurements

Mussel ropes were detached from the timber beams of the raft and lift with the assistance of a crane towed by a boat. Bivalves were separated from the ropes by cutting the byssal threads. Adult *Mytilus galloprovincialis* were selected to obtain individuals with homogenous shell length to ensure that any biochemical or biometric differences were not size-dependent. Individuals were measured along the anterior-posterior axis to the nearest 0.1 mm using Vernier callipers. Mussels were dissected and the mantle and the digestive gland were excised and placed separately in a glass vial for further biochemical determinations. Three replicate samples were obtained from each organ during each seasonal campaign, the replicates consisting of the organs of 3-4 mussels. Organs were immediately transported to the lab on ice and frozen at -50 °C to prevent enzymatic reactions, until lyophilization with a freeze-dryer (Ilshin Lab Co. Ltd, Korea). Tissues were then stored at -20 °C until homogenization with an ultrasonic Branson Sonifier (250/450 USA) for biochemical analysis. Three triplicate aliquots of the powered samples were dried and combusted to calculate the organic content (OC) of mussels' tissues.

Determinations of the Condition Index (CI, %), shell length (L, mm), tissue (DWt, mg) and shell (DWs, mg) dry weight were performed on a total of 180 individuals per site. Body tissues were excised from the shell valves. Samples were dried separately in pre-weighed aluminium containers at 110 °C for 24 h until constant weight to determine the dry shell weight (DWs) and the dry tissue weight (DWt). The CI was calculated according to Freeman (1974): CI= (DWt/DWs) x 100.

## 2.4. Biochemical composition

Protein content was calculated following Lowry et al. (1951), after alkaline hydrolysis with 0.5 NaOH at 30°C for 24 h. Carbohydrates were quantified according to the phenol-sulphuric acid method (Strickland and Parsons, 1968) using glucose as the standard. Glycogen was estimated with the same assay as carbohydrates after precipitation with 100% ethanol. Lipids were extracted following the method of Bligh and Dyer (1959) modified by Fernández-Reiriz et al. (1989). Total lipids were colorimetrically determined and tripalmitin (Sigma Aldrich Inc., Buchs, Switzerland) was used as a standard (Marsh and Weinstein, 1966).

Results for each biochemical component were expressed as relative percentage of dry weight (%DW) and as energy content in terms of Joules $l^{-1}$ (seston) or Joules $mg^{-1}$ (mussel samples). Energy conversion factors for proteins (18.00 J $mg^{-1}$), lipid (35.24 J $mg^{-1}$) and carbohydrate (17.16 J $mg^{-1}$) were obtained from Beukema and De Bruin (1979).

## 2.5. Statistical analysis

The seasonal and spatial differences in biochemical composition and biometric measurements were analyzed with a two-way analysis of variance (ANOVA) followed by Tukey's HSD test. Assumptions of normality and homogeneity of variances were tested *prior* to ANOVA with Shapiro-Wilk and Levene's test, respectively. Seasonal variations in shell length (L) were not considered for statistical analysis, since the available size at each season could vary as a

function of the commercial stage of the culture in the shellfish farm. Pearson's correlation coefficients (r) were calculated to determine the relationships between the Condition Index and the biochemical substrates present in the seston, mantle and the digestive gland. Statistical analyses were carried out using STATISTICA 7.0 software (StatSoft Inc) and graphs were plotted with the software R-project version 2.15.2.

# 3. Results

## 3.1. Physico-chemical conditions and biochemical composition of the diet

Two-way ANOVA showed that seasonality was the only significant effect for the physico-chemical measurements (Fig. 2). Temperature maxima was reached in the summer ($17.25 \pm 1.03$ ºC) and minima in winter ($13.20 \pm 0.36$ ºC) (Fig. 2; ANOVA, $F_{(4,49)}=44.72$, $P<0.001$). Slightly, but significantly higher pH values were recorded in winter ($8.17 \pm 0.19$) in comparison with autumns 2010 and 2011 ($7.86 \pm 0.14$) (Fig. 2; ANOVA, $F_{(4,49)}=12.41$, $P<0.001$).
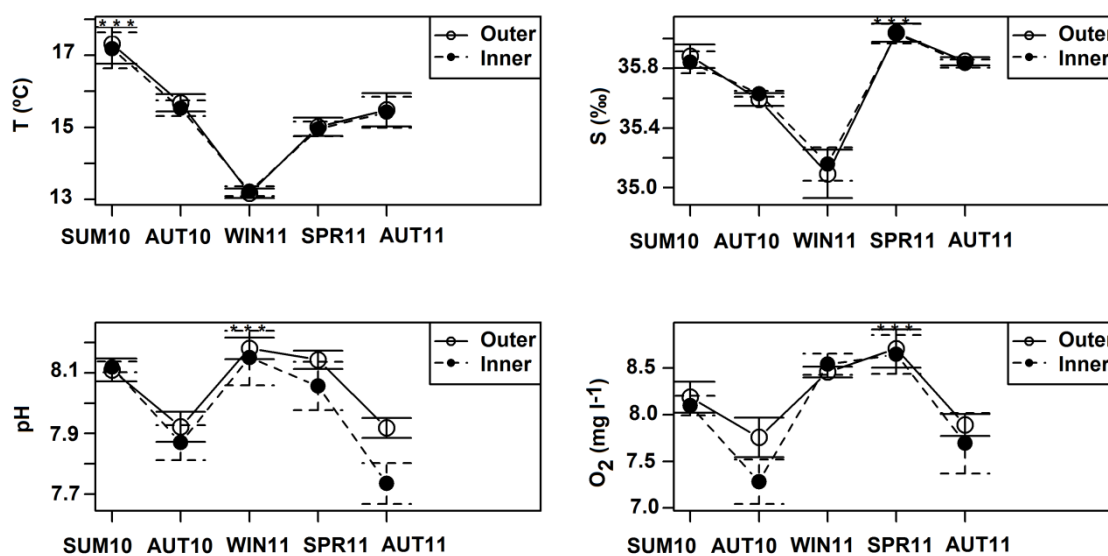


Fig. 2. Seasonal variations of the temperature (T, ºC), salinity (‰), pH and $O_2$ (mg $l^{-1}$) at the mussel raft situated in the outer and inner raft stations of the polygon. Each seasonal data point is based on the average of four weekly samples. Significant seasonal differences are denoted by *** $P <0.001$.

Salinity ($F_{(4,49)}$=32.46, $P$<0.001) and oxygen ($F_{(4,49)}$=13.66, $P$<0.001) peaked in spring (36.04 ± 0.15 ‰ and 8.68 ± 0.48 mg $l^{-1}$ $O_2$) and descended during both autumns (7.66 ± 0.57 mg $l^{-1}$ $O_2$) and winter (35.13 ± 0.15 ‰) (Fig. 2). Seston had an average of 62.68 ± 2.57 %POM, 37.32 ± 25.71 % ash and 5.87 ± 1.15 J $l^{-1}$ during the entire experimental period (Table 1). Proteins accounted as the major component during all the experiment (average 24.26 ± 13.36 % DW), after carbohydrates (average 10.40 ± 5.51 % DW) and lipids (average 10.31 ± 4.87 % DW) (Table 1). The analysis of variance revealed a significant effect of the season, site and the interaction term (site x season) on the biochemical composition of the seston (Table 3). The *post-hoc* test revealed that %POM, total energy content and biochemical substrates contained in the seston at both rafts showed the highest values during spring and the lowest values during winter (Tukey's HSD, $P$<0.05; Fig. 3 A, D). Seston from the outer raft showed higher %POM, proteins, carbohydrates and lipids than the inner raft during both autumns and winter (Tukey's HSD, $P$<0.05; Table 1; Fig. 3 A, D). However, seston at the outer raft showed a significantly lower energy content than that of the inner raft during autumn 2011 and winter (Tukey's HSD, $P$<0.05; Table 1; Fig. 3 A).

### 3.2. Biochemical composition of mussels' organs

Two-way ANOVA showed a significant effect of the season, site and their interaction term (site x season) on the biochemical composition of the mantle (Table 4). The average OC and energy content of the mantle tissue sampled at both mussel rafts showed maximum values during summer, autumn 2010 and 2011, while these parameters were substantially reduced during winter (Tukey's HSD, $P$<0.05; Fig. 3 B). The mantle of mussels from both sites had the highest proteins and lipids reserves in winter, when these components averaged 52 and 30 % of the mantle's energy reserves, respectively (Tukey's HSD, $P$<0.05; Fig. 4 A). On the other hand, proteins and lipids were largely depleted in the summer and only constituted 31 % and 18 % of the energy content of the mantle, respectively (Tukey's HSD, $P$<0.05; Fig. 4 A).

| Season | Site | POM (%) | Ash (%) | Protein %DW | Protein $J\ l^{-1}$ | Carbohydrates %DW | Carbohydrates $J\ l^{-1}$ | Lipids %DW | Lipids $J\ l^{-1}$ | Total energy $Jl^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Summer 2010 | Outer | 66.32 ± 6.12 | 33.68 ± 6.12 | 26.68 ± 3.39 | 2.48 ± 0.1 | 12.10 ± 1.05 | 1.07 ± 0.08 | 10.33 ± 1.70 | 1.87 ± 0.14 | 5.44 ± 0.26 |
| | Inner | 70.26 ± 2.18 | 29.74 ± 2.17 | 24.99 ± 6.91 | 2.32 ± 0.27 | 12.97 ± 1.74 | 1.17 ± 0.14 | 9.36 ± 0.94 | 1.74 ± 0.21 | 5.25 ± 0.04 |
| Autumn 2010 | Outer | 64.48 ± 0.29*** | 35.52 ± 0.29 | 27.47 ± 6.21*** | 2.54 ± 0.34 | 10.48 ± 1.66*** | 0.93 ± 0.14 | 10.29 ± 1.82*** | 1.87 ± 0.17 | 5.36 ± 0.51** |
| | Inner | 53.21 ± 3.09 | 46.79 ± 3.09*** | 18.28 ± 2.90 | 2.07 ± 0.14 | 7.03 ± 1.15 | 0.75 ± 0.03 | 8.20 ± 1.22 | 1.82 ± 0.1 | 4.66 ± 0.12 |
| Winter 2011 | Outer | 28.71 ± 1.51*** | 71.29 ± 1.51 | 10.12 ± 1.31*** | 1.98 ± 0.58 | 2.86 ± 0.18 | 0.87 ± 0.39 | 5.02 ± 0.23*** | 1.90 ± 0.31 | 4.76 ± 0.25 |
| | Inner | 21.45 ± 0.96 | 78.55 ± 0.96*** | 6.39 ± 0.69 | 2.72 ± 0.33 | 4.62 ± 1.51 | 1.16 ± 0.08 | 3.40 ± 0.26 | 2.85 ± 0.36 | 6.75 ± 0.24** |
| Spring 2011 | Outer | 97.25 ± 1.63 | 2.75 ± 1.63 | 47.84 ± 11.57 | 3.29 ± 0.73 | 19.46 ± 5.13 | 1.29 ± 0.41 | 18.27 ± 0.65 | 2.47 ± 0.21 | 7.07 ± 0.27 |
| | Inner | 94.65 ± 0.61 | 5.35 ± 0.61 | 42.34 ± 2.17 | 3.41 ± 0.33 | 16.06 ± 2.61 | 1.23 ± 0.2 | 15.41 ± 0.69 | 2.43 ± 0.18 | 7.08 ± 0.26 |
| Autumn 2011 | Outer | 88.39 ± 5.80*** | 11.61 ± 5.80 | 24.19 ± 2.94*** | 1.81 ± 0.38 | 12.87 ± 3.72*** | 0.92 ± 0.31 | 16.17 ± 2.12*** | 2.37 ± 0.49 | 5.12 ± 0.31 |
| | Inner | 42.04 ± 3.54 | 57.91 ± 3.54*** | 14.32 ± 1.51 | 3.15 ± 0.32 | 5.56 ± 1.07 | 1.16 ± 0.16 | 6.59 ± 1.49 | 2.85 ± 0.6 | 7.17 ± 0.51** |
| Average entire experiment | | 62.68 ± 2.57 | 37.32 ± 25.71 | 24.26 ± 13.36 | 2.58 ± 0.63 | 10.40 ± 5.51 | 1.06 ± 0.26 | 10.31 ± 4.87 | 2.22 ± 0.49 | 5.87 ± 1.15 |

Table 1. Seasonal variations of the seston collected at the outer and inner rafts in terms of organic matter (%POM) and biochemical composition expressed as % dry weight (%DW) and energy content of biochemical components (Joules $l^{-1}$). Significant pairwise comparisons based on Tukey's HSD tests are denoted by *$P$<0.05, **$P$<0.01 and ***$P$<0.001.  The pairwise comparisons reported in this table referred to the differences found between sites for each season.

| | | OC (%) | | Proteins (J mg-1) | | Carbohydrates (J mg-1) | | Lipids (J mg-1) | | Total energy (J mg-1) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Season | Site | Mantle | Gland | Mantle | Gland | Mantle | Gland | Mantle | Gland | Mantle | Gland |
| Summer 2010 | Outer | 88.99 ± 0.14 | 89.44 ± 0.66 | 5.62 ± 0.34 | 7.82 ± 0.3 | 7.87 ± 0.78 | 3.37 ± 0.29 | 3.56 ± 0.43 | 7.78 ± 0.27 | 17.04 ± 0.04 | 18.96 ± 0.39 |
| | Inner | 89.08 ± 0.02 | 89.65 ± 0.72 | 5.11 ± 0.7 | 7.55 ± 0.3 | 9.24 ± 0.7 | 3.23 ± 0.25 | 2.60 ± 0.35 | 8.34 ± 1.11 | 16.95 ± 0.28 | 19.11 ± 0.63 |
| Autumn 2010 | Outer | 92.16 ± 0.87 | 91.82 ± 0.51 | 6.85 ± 0.48*** | 7.15 ± 0.2 | 5.36 ± 0.53 | 4.33 ± 0.29 | 4.52 ± 0.83** | 7.28 ± 0.39* | 16.73 ± 0.83 | 18.75 ± 0.14 |
| | Inner | 91.37 ± 0.61 | 90.69 ± 0.11 | 5.31 ± 0.31 | 7.05 ± 0.46 | 7.67 ± 0.93* | 4.7 ± 0.22 | 3.31 ± 0.61 | 6.79 ± 0.28 | 16.28 ± 0.50 | 18.53 ± 0.49 |
| Winter 2011 | Outer | 83.21 ± 1.14 | 83.63 ± 1.49 | 8.67 ± 0.28*** | 8.70 ± 0.27 | 2.11 ± 0.52 | 2.75 ± 0.12 | 4.84 ± 0.83 | 4.90 ± 0.28* | 15.61 ± 0.58 | 16.34 ± 0.48 |
| | Inner | 85.69 ± 2.10 | 85.40 ± 1.28 | 7.90 ± 0.65 | 8.44 ± 1.34 | 3.84 ± 1.94* | 3.96 ± 2.01 | 4.89 ± 0.50 | 4.64 ± 0.53 | 15.56 ± 1.38 | 17.04 ± 0.78 |
| Spring 2011 | Outer | 85.88 ± 0.34 | 89.90 ± 0.07 | 8.02 ± 0.71 | 7.07 ± 0.64 | 3.79 ± 0.65 | 2.59 ± 0.39 | 3.85 ± 0.55 | 7.91 ± 0.29* | 14.52 ± 0.84 | 15.66 ± 0.75 |
| | Inner | 85.46 ± 3.26 | 88.43 ± 0.57 | 6.88 ± 0.49 | 8.23 ± 0.28 | 2.60 ± 0.31 | 1.97 ± 0.11 | 5.86 ± 1.19** | 6.13 ± 0.16 | 16.48 ± 1.53 | 15.34 ± 0.20 |
| Autumn 2011 | Outer | 90.92 ± 2.32 | 92.16 ± 0.80 | 6.92 ± 0.83*** | 7.00 ± 0.31 | 5.65 ± 0.72 | 5.44 ± 0.28 | 3.82 ± 0.58** | 6.17 ± 0.98* | 16.40 ± 0.55 | 18.60 ± 1.01 |
| | Inner | 92.78 ± 1.35 | 92.73 ± 1.01 | 6.27 ± 0.19 | 6.9 ± 0.22 | 7.95 ± 0.29* | 6.58 ± 1.13 | 3.26 ± 0.56 | 5.23 ± 0.85 | 17.48 ± 0.37 | 18.70 ± 0.43 |
| Average entire experiment | | 88.55 ± 3.44 | 89.36 ± 2.90 | 6.76 ± 1.24 | 7.59 ± 0.78 | 5.61 ± 2.51 | 3.89 ± 1.50 | 4.05 ± 1.09 | 6.52 ± 1.37 | 16.41 ± 1.09 | 17.99 ± 1.15 |

Table 2. Seasonal variations of the organic content (OC, %) and energy content of biochemical components (Joules mg$^{-1}$) of the mantle and digestive gland of *Mytilus galloprovincialis* sampled at the outer and inner rafts of Lorbé raft polygon (Galicia, NW Spain). Significant pairwise comparisons based on Tukey's HSD tests are denoted by *$P<0.05$, **$P<0.01$ and ***$P<0.001$. The pairwise comparisons reported in this table referred to the differences found between sites for each season.

Carbohydrates peaked in summer, when they represented an average of 50 % of the total energy content of the mantle in mussels from both sites, whereas they dropped abruptly in winter (Tukey's HSD, $P<0.05$; Fig. 4 A). Glycogen made up an average of 76 % of the total carbohydrate reserves in the mantle from both rafts and paralleled the seasonal fluctuations of the carbohydrates (Fig. 3 F). Tukey's pairwise comparisons indicated that the mantle from mussels cultured at the outer raft accumulated higher proteins and lipids compared to mussels from the inner raft during both autumns and winter, excepting for no significant differences detected for the lipid levels in winter (Tukey's HSD, $P<0.05$; Table 2; Fig. 3 F). On the other hand, the mantle from mussels at the outer raft showed lower levels of carbohydrates compared with those of the inner raft during both autumns and winter (Tukey's HSD, $P<0.05$; Table 2; Fig. 3 F).

ANOVA showed a significant effect of the season on the biochemical composition of the digestive gland, while an effect of the season, site and the interaction term (site x season) was only observed for the lipid content (Table 5). The digestive gland of mussels from both sites showed peak OC and energy values during summer and autumn, while minimum levels were registered in winter (Tukey's HSD, $P<0.05$; Fig. 3 C). Proteins descended during autumn 2010 and 2011 in the gland of mussels from both sites and recovered in winter, when they constituted 40% of the gland's energy reserves (Tukey's HSD, $P<0.05$; Fig. 3 G and 4 B). The carbohydrates reserves found in the gland of mussels from both sites were reduced in spring and recovered during both autumns, when they represented an average of 28 % of the total energy of the gland (Tukey's HSD, $P<0.05$; Fig. 3 G and 4 B). Glycogen constituted an average of 35 % of the total carbohydrates of the gland and followed the seasonal fluctuations of the carbohydrates (Fig. 3 G). Lastly, the lipid content of the gland at both mussel rafts descended in winter and peaked in summer, contributing to 42 % of the gland's energy during this period (Tukey's HSD, $P<0.05$; Fig. 3 G and 4 B). The gland of mussels from the outer station exhibited significantly greater lipid content than the gland from mussels cultured at the inner

station during all seasons, excepting for no significant differences detected during the summer (Tukey's HSD, $P<0.05$; Table 2; Fig. 3 G).
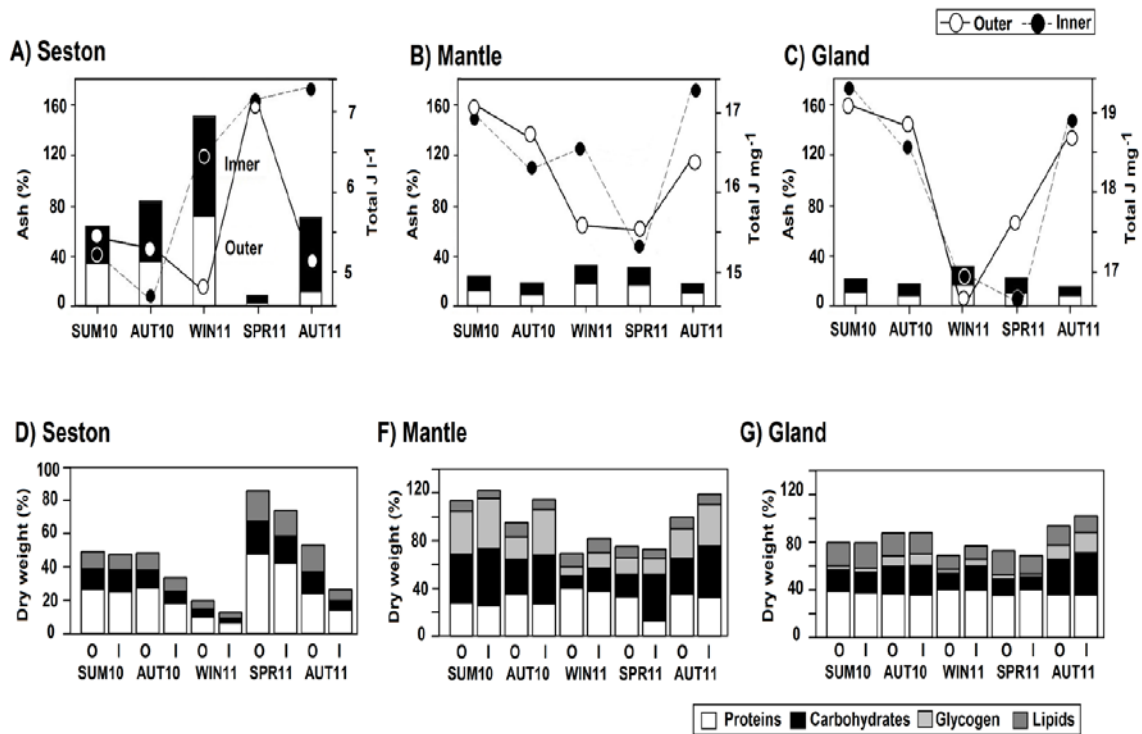


Fig. 3. Inorganic content (% ash; bars) and biochemical composition in terms of total energy (Joules; lines) and % dry weight (%DW) of: A, D) seston; B,F) mantle and C,G) digestive gland recorded during five different seasons at the raft situated in the outer (O) and inner (I) regions of the raft polygon.
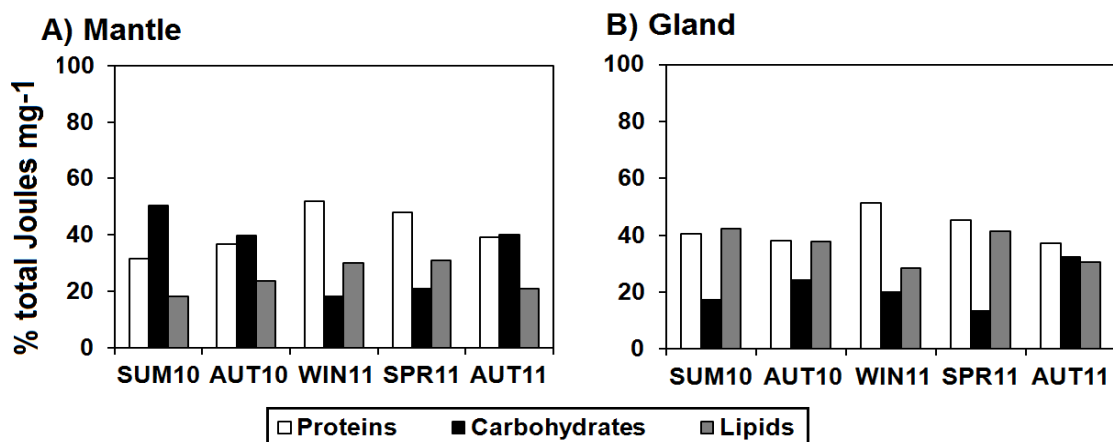


Fig. 4. Seasonal percentage contribution of the proteins, carbohydrates and lipids to the total energy content (Joules mg$^{-1}$) of the mantle (A) and the digestive gland (B).

| Effect | SS | MS | F-value (1,20) | P-value | SS | MS | F-value (1,20) | P-value |
|---|---|---|---|---|---|---|---|---|
| **% POM** | | | | | **Total energy (J l⁻¹)** | | | |
| Season | 15439.72 | 3859.93 | 370.43 | <0.001*** | 15.31 | 3.83 | 7.47 | <0.001*** |
| Site | 1209.42 | 1209.42 | 116.07 | <0.001*** | 3 | 3 | 5.86 | <0.001*** |
| Site x Season | 2309.22 | 577.3 | 55.4 | <0.001*** | 10.02 | 2.5 | 4.88 | <0.001*** |
| Error | 208.4 | 10.42 | | | 10.25 | 0.51 | | |
| | | | | | | | | |
| **Protein (%DW)** | | | | | **Protein (J l⁻¹)** | | | |
| Season | 4316.93 | 1079.23 | 41.8 | <0.001*** | 4.55 | 1.14 | 7.24 | <0.001*** |
| Site | 269.42 | 269.42 | 10.44 | <0.01** | 0.75 | 0.75 | 4.75 | <0.05* |
| Site x Season | 73.8 | 18.45 | 0.71 | <0.001*** | 3.16 | 0.79 | 5.03 | <0.01** |
| Error | 516.37 | 25.82 | | | 3.14 | 0.16 | | |
| | | | | | | | | |
| **Carbohydrates (%DW)** | | | | | **Carbohydrates (J l⁻¹)** | | | |
| Season | 643.38 | 160.85 | 27.41 | <0.001*** | 0.56 | 0.14 | 2.52 | <0.05* |
| Site | 67.82 | 67.82 | 11.56 | <0.01** | 0.05 | 0.05 | 0.82 | <0.05* |
| Site x Season | 53.21 | 13.3 | 2.27 | <0.05* | 0.24 | 0.06 | 1.07 | <0.05* |
| Error | 117.36 | 5.87 | | | 1.11 | 0.06 | | |
| | | | | | | | | |
| **Lipids (%DW)** | | | | | **Lipids (J l⁻¹)** | | | |
| Season | 494.02 | 123.5 | 75.66 | <0.001*** | 3.23 | 0.81 | 7.85 | <0.001*** |
| Site | 87.91 | 87.91 | 53.85 | <0.001*** | 0.43 | 0.43 | 4.18 | <0.05* |
| Site x Season | 73.81 | 18.45 | 11.3 | <0.001*** | 1.3 | 0.32 | 3.16 | <0.01** |
| Error | 32.65 | 1.63 | | | 2.05 | 0.1 | | |

Table 3. Results of the two-way ANOVA testing the influence of season, site and the interaction term (site x season) on the biochemical composition of the seston expressed in terms of % dry weight (%DW) and energy content (Joules l⁻¹). Significant differences are indicated by *$P$<0.05, **$P$<0.01, ***$P$<0.001 and ns (not significant).

| Effect | SS | MS | $F$-value (1,20) | $P$-value | SS | MS | $F$-value (1,20) | $P$-value |
|---|---|---|---|---|---|---|---|---|
| **OC (%)** | | | | | **Total Energy (J mg$^{-1}$)** | | | |
| Season | 279.21 | 69.8 | 28.11 | <0.001*** | 9.33 | 2.33 | 2.89 | <0.05* |
| Site | 3.12 | 3.12 | 1.26 | 0.27 ns | 3.7 | 3.7 | 4.58 | 0.06 ns |
| Site x Season | 12.46 | 3.12 | 1.25 | 0.32 ns | 5.66 | 1.42 | 1.75 | 0.17 ns |
| Error | 49.66 | 2.48 | | | 16.16 | 0.81 | | |
| | | | | | | | | |
| **Protein (%DW)** | | | | | **Protein (J mg$^{-1}$)** | | | |
| Season | 925.11 | 231.28 | 37.91 | <0.001*** | 31.44 | 7.86 | 26.49 | <0.001*** |
| Site | 384.89 | 384.89 | 63.09 | <0.001*** | 1.65 | 1.65 | 5.55 | <0.001*** |
| Site x Season | 346.55 | 86.64 | 14.2 | <0.001*** | 5.79 | 1.45 | 4.88 | <0.001*** |
| Error | 122.02 | 6.1 | | | 5.94 | 0.3 | | |
| | | | | | | | | |
| **Carbohydrates (%DW)** | | | | | **Carbohydrates (J mg$^{-1}$)** | | | |
| Season | 2927.35 | 731.84 | 32.787 | <0.001*** | 142.15 | 35.54 | 48.06 | <0.001*** |
| Site | 1127.53 | 1127.53 | 50.514 | <0.001*** | 12.75 | 12.75 | 17.25 | <0.001*** |
| Site x Season | 144.04 | 36.01 | 1.613 | <0.05* | 12.59 | 3.15 | 4.26 | <0.05* |
| Error | 446.42 | 22.32 | | | 14.79 | 0.74 | | |
| | | | | | | | | |
| **Lipids (%DW)** | | | | | **Lipids (J mg$^{-1}$)** | | | |
| Season | 54.53 | 13.63 | 5.51 | <0.01** | 15.14 | 3.79 | 8.07 | <0.001*** |
| Site | 19.69 | 19.69 | 7.96 | <0.01** | 0.13 | 0.13 | 0.28 | <0.05* |
| Site x Season | 11.49 | 2.87 | 1.16 | <0.05* | 9.96 | 2.49 | 5.3 | <0.01** |
| Error | 49.46 | 2.47 | | | 9.38 | 0.47 | | |

Table 4. Results of the two-way ANOVA testing the influence of season, site and the interaction term (site x season) on the biochemical composition of the mantle of mussel *M. galloprovincialis* in terms of % dry weight (%DW) and energy content (Joules mg$^{-1}$). Significant differences are indicated by *$P$<0.05, **$P$<0.01, ***$P$<0.001 and ns (not significant).

| Effect | SS | MS | $F$-value (1,20) | $P$-value | SS | MS | $F$-value (1,20) | $P$-value |
|---|---|---|---|---|---|---|---|---|
| *OC (%)* | | | | | *Total Joules mg$^{-1}$* | | | |
| Season | 220.04 | 55.01 | 77.51 | <0.001*** | 28.49 | 7.12 | 20.24 | <0.001*** |
| Site | 0.01 | 0.01 | 0.01 | 0.92 ns | 0.08 | 0.08 | 0.22 | 0.64ns |
| Site x Season | 9.97 | 2.49 | 3.51 | 0.07ns | 3.1 | 0.77 | 2.2 | 0.10ns |
| Error | 14.19 | 0.71 | | | 7.04 | 0.35 | | |
| | | | | | | | | |
| *Protein (%DW)* | | | | | *Protein (J mg$^{-1}$)* | | | |
| Season | 80.95 | 20.24 | 3.07 | <0.05* | 9.78 | 2.44 | 8.32 | <0.001*** |
| Site | 1.51 | 1.51 | 0.22 | 0.63 ns | 0.06 | 0.06 | 0.19 | 0.66 ns |
| Site x Season | 41.76 | 10.44 | 1.58 | 0.21ns | 2.17 | 0.54 | 1.85 | 0.15 ns |
| Error | 131.92 | 6.6 | | | 5.88 | 0.29 | | |
| | | | | | | | | |
| *Carbohydrates (%DW)* | | | | | *Carbohydrates (J mg$^{-1}$)* | | | |
| Season | 1524.57 | 381.14 | 24.09 | <0.001*** | 48.65 | 12.16 | 20.85 | <0.001*** |
| Site | 32.33 | 32.33 | 2.04 | 0.16 ns | 1.17 | 1.17 | 2 | 0.17ns |
| Site x Season | 111.76 | 27.94 | 1.77 | 0.17 ns | 3.8 | 0.95 | 1.63 | 0.20 ns |
| Error | 316.47 | 15.82 | | | 11.66 | 0.58 | | |
| | | | | | | | | |
| *Lipids (%DW)* | | | | | *Lipids (J mg$^{-1}$)* | | | |
| Season | 290.82 | 72.7 | 28.24 | <0.001*** | 39.81 | 9.95 | 26.95 | <0.001*** |
| Site | 17.05 | 17.05 | 6.62 | <0.01** | 2.55 | 2.55 | 6.9 | <0.01** |
| Site x Season | 32.37 | 8.09 | 3.14 | <0.05* | 4.44 | 1.11 | 3.01 | <0.05* |
| Error | 51.49 | 2.57 | | | 7.38 | 0.37 | | |

Table 5. Results of the two-way ANOVA testing the influence of season, site and the interaction term (site x season) on the biochemical composition of the digestive gland of mussel *M. galloprovincialis* in terms of % dry weight (%DW) and energy content (Joules mg$^{-1}$). Significant differences are indicated by *$P$<0.05, **$P$<0.01, ***$P$<0.001 and ns (not significant).

### 3.3. Condition Index and biometric measurements

The variations in Condition Index (CI), shell length (L), tissue and shell dry weight (DWt, DWs) are illustrated in Fig. 5. L and DWs did not differ significantly among rafts, ensuring that any differences in meat yield were not a function of site-specific L variations (ANOVA, $P>0.05$). The CI was significantly higher in autumn at both rafts, declined in winter, recovered slightly in spring and declined again in summer in mussels from both rafts (Tukey's HSD, $P<0.001$; Fig. 5). The DWt and DWs followed an analogous annual cycle. Despite the fact that the CI displayed a similar seasonal pattern at both rafts, two-way ANOVA followed by *post-hoc* testing indicated that mussels from the outer raft showed a higher CI and DWt than innermost individuals during both autumns and winter (Tukey's HSD, $P<0.001$; Fig. 5).

 A moderate correlation was obtained between the CI and the organic ($r=0.375$, $P<0.01$) and lipid ($r=0.399$, $P<0.01$) content of the seston. A highly significant correlation was established between the CI and the OC of the mantle ($r=0.524$, $P<0.01$) and the gland ($r=0.704$, $P<0.001$). The CI was also significantly correlated to the protein ($r=0.488$, $P<0.01$), carbohydrate ($r=0.478$, $P<0.01$) and glycogen ($r=0.533$, $P<0.01$) in the digestive gland.
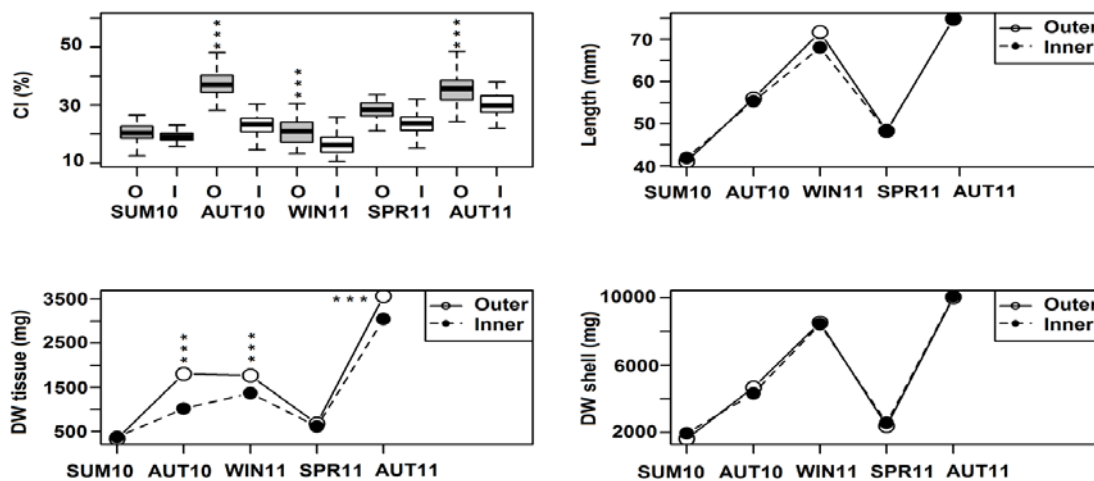


Fig. 5. Condition Index (CI, %), shell length (L, mm), tissue and shell dry weight (DW, mg) of mussels *Mytilus galloprovincialis* obtained during five different seasons at the raft situated in the outer (O) and inner (I) region of the raft polygon. Significant differences are denoted by ***$P$ <0.001.

## 4. Discussion

Relatively few studies have focused on the variations of proteins, lipids and carbohydrates of individual organs of bivalves, as most effort has been diverted to the biochemical composition of the bulk tissue of bivalves exposed to natural diets (Okumus and Stirling, 1998; Freites et al., 2003; Orban et al., 2002; 2006; Çelik et al., 2012; Karayucel et al., 2013; Pogoda et al., 2013; Gallardi et al., 2014) or laboratory conditions (Pérez-Camacho et al., 2003; Albentosa et al., 2007; Fernández-Reiriz et al., 2007; Gallardi et al., 2014). However, the separate analysis of the biochemical composition of the mantle and the gland carried out in this study highlighted tissue-specific differences in the seasonal cycle of storage and expenditure of reserves.

Proteins are considered the main energy substrate during gamete development (Zandee et al., 1980; Ren et al., 2003; Marin et al., 2003; Baek et al, 2014) and peak and lowest levels in the bulk tissue of *M. galloprovincialis* are known to be coincident with the main periods of gamete development (winter) and spawning (spring), respectively (Freites et al., 2003; Çelik et al., 2012; Karayucel et al., 2013). Similarly, decreases in lipid content in the bulk tissue of mussels have also been associated with gamete formation and spawning effort in winter-spring, whereas peak values coincide with the phytoplankton bloom in spring-summer (Zandee et al., 1980; Freites et al., 2003; Narváez et al., 2008; Prato et al., 2010; Çelik et al., 2012; Suárez et al., 2013; Karayucel et al., 2013; Gallardi et al., 2014). Nonetheless, in this study, the individual analysis of both tissues revealed that proteins were first depleted in the gland during autumn, while the mantle maintained high levels of proteins until summer. Likewise, lipids were highest in the mantle during winter and decreased in summer, following the typical mass spawning period in spring (Villalba, 1995; Peteiro et al., 2011). In contrast, the lipid stock in the gland revealed an opposite pattern. These distinct patterns of storage and utilization of lipids and proteins might be related to the distinct physiological functions of each organ. The mantle is the tissue that supports gonadal development, while the digestive gland is responsible for the storage of metabolic reserves, the mobilization of reserves during periods of physiological stress (i.e. food shortage or gametogenesis) and intracellular digestion and absorption of the food

previously digested in the stomach (Zandee et al., 1980; Cartier et al., 2004). Thus, proteins and lipids might have been transferred from the gland to the mantle for gamete production during winter downwelling and stored in the gland during the summer upwelling (Caers et al., 1999; 2003; Martínez-Pita et al., 2012). The double amounts of lipids measured for the digestive gland (16.56 % DW) with respect to those of the mantle (9.48% DW) confirmed that the gland plays a central role as a storage tissue of lipids (Zandee et al., 1980; Martínez-Pita et al., 2012; Karnjanapratum et al., 2013).

Proteins and lipids fluctuated in an opposite fashion with respect to carbohydrates (Zandee et al., 1980; Ren et al., 2003; Marin et al., 2003). This opposite relationship was more patent in the mantle because it is the principal storage organ of glycogen in the vesicular and adipogranular cells (Zandee et al., 1980; Bayne et al., 1982; Mathieu & Lubet, 1993) and, in fact, it contained a two-fold higher amount of glycogen than the gland. Glycogen represents more than 50 % of the total carbohydrate reserves in bivalves and followed the same pattern as the total carbohydrate reserves (Zandee et al., 1980; Park et al., 2011; Baek et al., 2014). Glycogen was stored in the mantle during summer and exhausted during winter downwelling, when seston %POM was minimal, but lipid reserves in the mantle reached their seasonal peak. Thus, it is probable that glycogen reserves were being converted to lipids by the enzyme glycogen phosphorylase to support vitellogenesis (San Juan Serrano et al., 1998). Martínez-Pita et al. (2012) also reported the expenditure of glycogen in the mantle of *M. galloprovincialis* during the ripeness stage in winter. Accordingly, other studies highlighted this inverse correlation, suggesting that glycogen is converted to lipids to fuel gametogenesis (Pogoda et al., 2013; Gallardi et al., 2014). Similar seasonal trends in glycogen accumulation were described for the bulk tissue of mussels, clams and oysters (Zandee et al., 1980; Kang et al., 2000; Freites et al., 2003; Marin et al., 2003).


The seasonal variations in Condition Index (CI) were coincident with that of *M. galloprovincialis* cultured in the Ría de Vigo (Zuñiga et al., 2013). The CI was significantly correlated with the nutritional characteristics of the diet and with the cycles of accumulation and

depletion of reserves in the mussel organs. A close correlation between the seasonal fluctuations in the CI and the variations in the biochemical reserves has also been found for several species of bivalve mollusks (Okumus and Stirling, 1998; Orban et al., 2002; 2006; Park et al., 2011; Baek et al., 2014). During winter, the significant decreases in lipids and glycogen reserves in the digestive gland and the mantle, respectively, were followed by a concomitant decline in the CI and DWt. This presumably owed to the high energy investment during the typical winter gonadal development (Villalba, 1995) and to the significantly low food quality (4-6 J $l^{-1}$, 21-28 % POM) and chlorophyll levels measured during winter downwelling (Irisarri et al., 2014 a). The expenditure of biochemical reserves was further corroborated at a physiological level, as the Scope for Growth –the energy available for growth and reproduction– was significantly lower during the winter downwelling (-1 to 4 J $h^{-1}$) than during the spring bloom (12 to 13 J $h^{-1}$). In this way, our results corroborated that the nutritional value of the seston was lowest during the winter downwelling (4-6 J $l^{-1}$, 21-28 % POM) and highest during the spring bloom (7 J $l^{-1}$, 94-97 % POM), when cold (15ºC) upwelled nutrient-rich waters typically enter the Ría and increase primary productivity (Figueiras et al., 2002; Irisarri et al., 2014 a). Similarly, clams displayed an accumulation of reserves, coupled with a positive energy balance, when conditioned at low temperatures (14ºC) without food stress (Férnandez-Reiriz et al., 2007). Following the typical massive spawning event in spring, it is likely that the protein and lipid reserves of the mantle were exhausted during the gonadal restoration that usually occurs in the summer (Villalba, 1995). The summer is typically characterized by a succession of blooms and thermal stratification episodes (Varela et al., 2001; Figueiras et al., 2002). In fact, the significantly higher water temperature (17 ºC) and the depletion of chlorophyll were indicators of a summer thermocline during our study (Irisarri et al., 2014 a). These stressful environmental conditions reduced the energy intake and intensified the metabolic expenditure, resulting in 50% lower DWt and negative Scope for Growth (-24 to -45 J $h^{-1}$) (Irisarri et al., 2014 a). Similarly, clams used their energy reserves to compensate for the high metabolic expenditure and the negative energy balance when conditioned at high temperatures (18ºC) with low food ration (Fernández-Reiriz et al., 2007). In contrast, mussels in autumn showed a higher CI and 4 to 9

times greater DWt than in summer, which could be associated with the accumulation of glycogen in the mantle throughout the intermittent upwelling episodes that precede the stratification events (Varela et al., 2001; Figueiras et al., 2002). This was also in good agreement with the higher Scope for Growth computed for the autumn bloom (10 to 18 J $h^{-1}$) (Irisarri et al., 2014 a). Thus, our results suggested that mussels harvested *prior* or shortly after spawning in spring and summer would be more suitable for processing as canned or frozen shellfish, while the higher CI and DWt during autumn and winter suggests that mussels harvested during this period are excellent for fresh consumption.

Our results agreed well with previous studies that reported that suspension-feeding bivalves experience site-specific variations in quantity and quality of food as a result of natural fluctuations of the seston over short (resuspension events, tidal cycle) or long (seasonal) time scales (Iglesias et al., 1996; Norkko and Thrush, 2006; Waite et al., 2005; Pérez-Camacho et al., 2014; Hernández-Otero et al., 2014), or as a consequence of human activities that reduce food organic loads and chlorophyll levels compared to neighboring sites (Marin et al., 2003; Baek et al., 2014). In fact, spatial differences in bivalve biochemical composition and CI (Férnandez-Reiriz et al., 1996; Marin et al., 2003; Norkko and Thrush, 2006; Baek et al., 2014), feeding physiology (Navarro et al., 1991; Iglesias et al., 1996) and growth rates (Pérez-Camacho et al., 1995; 2014; Waite et al., 2005; Hernández-Otero et al., 2014) have been linked to variability in food quantity (i.e. chlorophyll-a, suspended matter concentration) and quality (i.e. food energy, %POM) between outer and inner regions of the same coastal embayment. Bivalves in this study experienced significant short-term spatial fluctuations in the nutritional value of the seston during autumn and winter. Reductions in seston quality at the innermost site were recorded during stormy conditions, when intensified current velocities resuspended inorganic sediments and resulted in a dilution of 17 to 50 % of the POM available for filter-feeders at this shallow raft (Irisarri et al., 2013; Zuñiga et al., 2014). During this period, we also observed that seston's energy content at the inner raft surpassed that of the outer raft, so it is likely that refractory allocthonous material was being mixed in the water column during the storms (Zuñiga et al.,

2014). Nonetheless, this refractory material was probably not included in the mussels' diet, as previous studies documented their limited ability to ingest and absorb refractory lignocellulosic material (Kreeger et al., 1988; 1990). These increments in the turbidity of the feeding environment were manifested at physiological and biochemical levels in the bivalves cultured at the inner raft. Mussels enhanced the clearance rate to compensate for short-term reductions in the absorption of organic nutrients in the gut during resuspension events (Irisarri et al., 2013; 2014). This physiological plasticity in the feeding response was translated into a similar Scope for Growth (i.e. energy available for growth and reproduction) for mussels at both rafts (Irisarri et al., 2014 a). Accordingly, although stressful feeding conditions corresponded to temporal reductions in Condition Index, proteins and lipids reserves of innermost mussels, we found a similar energetic content in the mantle and the gland among sites. Thus, the different timing in accumulation of reserves was probably temporary and was not recorded outside the short-term resuspension events. Furthermore, mussels showed similar monthly growth rates and CI from the onset (March) to the end (November) of an experimental culture at the same outer and inner rafts monitored in this study (Irisarri et al., 2014 b). Interestingly, Fernández-Reiriz et al. (1996) found that mussels from Arnela, the innermost raft polygon of the Ría Ares-Betanzos (see Fig. 1), had greater CI and glycogen levels than Lorbé. However, it is probable that the lower mussel production in Arnela exerted a lower pressure in the standing stock of phytoplankton than mussels in Lorbé (Fernández-Reiriz et al., 1996). Overall, the findings of the present study, in combination with physiological and biometric investigations, emphasize that the spatial differences in feeding conditions did not affect bivalves over a long-time scale, given the similar seasonal variability in seston quantity, quality and chlorophyll among sites (Irisarri et al., 2013; 2014 a, b).

In summary, we concluded that the seasonal variations in the proximate composition and CI of mussels *M. galloprovincialis* were closely linked to the natural fluctuations in the composition of the diet and the different stages of the annual reproductive cycle described for the Galician Rías. Bivalves at the innermost raft were exposed to reductions in seston quality during storm-

induced resuspension events in autumn-winter. These short-term increases in turbidity did not seem to significantly affect bivalves' proximate composition and meat yield over a longer time scale, indicating that both the inner and outer regions of the raft polygon are suitable sites for the suspended culture of bivalves.

# Chapter 8

General discussion and conclusions

This study evaluated the seasonal and spatial variability of the Scope for Growth, proximate composition, fatty acid signature and growth of mussels cultured in two different ecosystems, the Ría Ares-Betanzos (Galicia, Spain) and the Passamaquoddy region of the Bay of Fundy (Canada). The first major objective was to analyze the seasonal variations in mussels' physiological, biochemical and molecular responses. The second objective was to determine if mussels cultured near a fish farm enhanced their performance compared to mussels cultured at a reference site distant from the fish cages. The temporal and spatial variations in seston' quality and quantity were also investigated, to analyze natural dietary fluctuations and to assess if any particulate or dissolved fish wastes could have enhanced seston loads and chlorophyll biomass, respectively. The study in Ría Ares-Betanzos was conducted in two mussel (*Mytilus galloprovincialis*) rafts of the Lorbé raft polygon during five different seasons. The first raft was placed in the innermost region of the raft polygon, in the proximity (170 m North) of a red sea bream (*Pagellus bogaraveo*) fish farm. The second raft was moored in the outer region of the raft polygon, 550 m North from the net pens. The study in the Bay of Fundy was performed during the summer (June) in an IMTA and monoculture sites. The IMTA site consisted of a commercial mussel (*Mytilus edulis*) raft moored adjacent to a salmon cage array. The monoculture mussel farm was 8.5 km away from the IMTA site.

The results obtained in Ría Ares-Betanzos showed that mussels cultured in suspension at both rafts were subjected to seasonal variations in the suspended organic matter loads, chlorophyll, proteins, carbohydrates and lipids contained in the seston. The variations in the feeding environment were closely associated with periods of high and low primary productivity during upwelling and downwelling events, respectively. Maximum seston quality (0.37-0.42 mg $l^{-1}$ POM), chlorophyll levels (0.86-1 µg $l^{-1}$) and amount of biochemical components of the seston (44.9% proteins, 17.7% carbohydrates, 16.8 % lipids) occurred in spring, when upwelling processes reached the highest intensity in the Galician Rías. In contrast, the lowest quality (24 % POM) and amount of proteins (8.2%DW), carbohydrates (17.7%DW) and lipids (16.8 %DW) in the seston coincided with the winter downwelling period, although some chlorophyll was still

available for filter-feeders ($>0.6$ µg l$^{-1}$) as a result of the vertical mixing of the surface with deeper layers. In fact, minimum chlorophyll levels were recorded during the intermittent summer stratification events (0.38-0.51 µg l$^{-1}$). During this period, it is probable that high solar radiation created a deeper thermocline layer that prevented colder and nutrient-reach deeper layers to be upwelled towards surface waters. This would explain the reduced chlorophyll levels measured during the summer, as limited nutrients could not sustain a high phytoplankton biomass. The duration of these intermittent periods of upwelling-relaxation-thermal stratification have been documented to be subjected to high inter-annual variability, depending on the intensity and direction of the wind (Figueiras et al., 2002; Álvarez-Salgado et al., 2008; Villegas-Ríos et al., 2011). Thus, the variable duration of the stratification events seemed to affect the intensity of the autumn bloom, since chlorophyll and POM loads were significantly higher in autumn 2011 (0.67 µg l$^{-1}$ and 0.43 mg l$^{-1}$) than in autumn 2010 (0.53 µg l$^{-1}$ and 0.33 mg l$^{-1}$). Furthermore, the amount of chlorophyll contained in each phytoplankton size-fraction and the analysis of the fatty acid biomarkers of the seston reflected the succession of two main types of phytoplankton throughout the annual upwelling- downwelling pulses. The upwelling of cold and nutrient-rich waters during spring-summer induced the occurrence of a bloom of nanophytoplankton (2-20 µm). This size-fraction was the most abundant during the whole annual cycle and comprised 70 % of the total chlorophyll in spring-summer. The analysis of the fatty acid biomarkers of the seston revealed that the abundance of nanophytoplankton owed to a bloom of diatoms. However, the composition and structure of the phytoplankton community shifted during winter, when downwelling favourable winds prevailed and cold nutrient-rich waters were replaced by warmer nutrient-poor waters. During this period, there was an occurrence of a dinoflagellate bloom within the micro- ($>20$ µm) and picophytoplankton (0.2-2 µm) size-fractions, which became more relevant and constituted 50% of the total chlorophyll. Concurrently, other studies carried out in the Galician Rías found that the phytoplankton size-structure has a different composition during the upwelling and downwelling seasons. During upwelling events, large-sized microphytoplankton is the most abundant, although nanophytoplankton might also constitute a large fraction of the total primary production

(Figueiras et al., 2002; Cermeño et al., 2006; Froján et al., 2014; Figueiras et al., 2014). The picophytoplankton fraction is always present with very low abundance during the upwelling season, but increases its importance during downwelling events as observed in our study (Figueiras et al., 2002; Cermeño et al., 2006; Arbones et al., 2008; Froján et al., 2014; Figueiras et al., 2014).

The seasonal fluctuations in food quality, quantity and seston composition were reflected at both the physiological –Condition Index, feeding, digestive and metabolic rates– and biochemical –proximate composition and fatty acid signature – levels of biological organization of the mussels cultured in suspension in Ría Ares-Betanzos.

The Scope for Growth (SFG) is a conceptual model that integrates the results of the feeding rates (clearance rate and organic ingestion rate), digestive rates (absorption efficiency and absorption rate) and metabolic rates (oxygen consumption and ammonia excretion rate) of an animal in order to estimate the energy available for growth and reproduction. Thus, the SFG measures the difference between the energy gains from the absorbed ratio and the metabolic losses through respiration and excretion. When the energy gains exceed the total metabolic losses, an organism can allocate energy for somatic growth and reproduction, resulting in a positive net energy balance or SFG. To model the SFG it is necessary to measure the former physiological rates and to know the dietary (amount of chlorophyll, POM, PIM, TPM) and environmental (temperature, salinity, oxygen) characteristics under which these physiological rates were obtained. This is especially relevant when the SFG is directly calculated from measurements obtained in the field, where, unlike the case of laboratory studies, environmental conditions vary quickly within a large natural range. The interacting effect of the food availability and temperature are considered the most influential factors that condition the overall energy budget (see review by Bayne and Newell, 1983). Some bivalves experience a negative SFG at very low or elevated temperatures, although the range of temperatures over which the SFG remains constant differs between species (Bayne and Newell, 1983). Nevertheless, food quality and quantity is best thought as the most important environmental factor limiting the

overall SFG of bivalves growing in their natural marine environment (Bayne and Newell, 1983; Pérez-Camacho et al., 1995; Wong and Cheung, 2001; 2003; Helson and Gardner, 2007).

Bivalves acquire their energy by clearing the particles suspended in the water. Particles are transported by ciliary currents along the gills towards the labial palps. The volume of water cleared of particles per unit time is referred as the clearance rate (CR). The CR of mussels cultured in Ría Ares-Betanzos was positively correlated with the amount of chlorophyll and seston quality ($f$ =POM/TPM) and negatively correlated with PIM. In this way, mussels decreased the CR during periods of low food quality like winter, when high inorganic seston loads 'diluted' the quality of the seston, but increased the CR with the chlorophyll bloom in spring (2.3 and 3 lh$^{-1}$, respectively). A review of the literature on the effects of particles loads on the CR also shows that it declines below or above a threshold of particle concentration, even if this threshold varies between species (Bayne and Widdows, 1978; Widdows et al., 1979; MacDonald and Ward, 1994; Hawkins et al., 1999; Riisgård, 2001; Velasco and Navarro, 2005; Filgueira et al., 2009). When the concentration of particles increases, bivalves generally regulate the amount of food ingested by reducing the clearance rate and producing pseudofeces. In this sense, the organic ingestion rate (OIR) followed the pattern of the CR, since seston loads in the Galician Rías are below the threshold for pseudofeces production (<5 mg l$^{-1}$ TPM). Although the variation of the CR and OIR were best explained by the total amount of chlorophyll available in the water column (74-86% and 77-87% of the variability, respectively), nanophytoplankton and microphytoplankton were the size-fractions that explained the highest percent variation of the former feeding rates. This suggested that nano- and microphytoplankton were selectively cleared and ingested by the mussels in comparison with the pico- size-fraction. Concurrently, Froján et al. (2014) showed that mussels cultured in Ría de Vigo modified the structure of the plankton community, suggesting that mussels exert a top-down control over the micro- and nanoplankton community without affecting the picoplankton size-fraction. Similarly, previous studies suggested that bivalves have size-specific clearance rate (Fournier et al., 2012), particle retention efficiency (Strohmeier et al., 2012) and depletion rates (Cranford et al., 2014). Fournier et al. (2012) studied the clearance rate of pearl oysters *Pinctada*

*margaritifera* with a flow through chamber method and observed how the CR increased with the size of plankton. In general, pearl oysters cleared chlorophyll >2 µm at higher rate than chlorophyll <2 µm and the clearance rate of picoplankton by pearl oysters was extremely low compared to clearance of nanoplankton and microplankton, as observed in our study. A study developed by Cranford et al. (2014) suggested that phytoplankton depletion is size-specific and in the high-density mussel culturing area of Lorbé polygon food depletion was highest for particles in the 4 to 45 µm size range (nano- and microplankton). Strohmeier et al. (2012) observed that mussels' retention efficiency (RE) increased progressively from small to large particles. Generally, mussels showed the highest RE for particles between 30 to 35 µm, which was in agreement with the preferential clearance and ingestion of the microphytoplankton size-class observed in our study. However, mussels displayed a physiological regulation on the retention mechanisms of the gills upon changes in ambient particle distribution (Strohmeier et al., 2012). When small particles dominated, mussels showed the maximum RE for particles between 7 and 15 µm diameter (i.e. nanophytoplankton size-class in our study), albeit they also proved to have some capacity to retain particles ranging between 1 to 4 µm diameter (Strohmeier et al., 2012).

The absorption efficiency (AE) estimates the percent of the organic ingested ratio that is absorbed in the gut. The food quality (*f*) explained the highest variability of the absorption efficiency recorded for mussels at both rafts (94-98 % variability). In fact, the best model was obtained when the AE was plotted against the inverse transformation of the food quality (1/*f*), because this transformation linearized the hyperbolic trend previously observed between these two variables (Cranford and Hill, 1999; Hawkins et al., 1996; Navarro et al., 1996). Thus, the maximum absorption efficiency (94-97 %) was observed during the spring bloom (*f*=0.95 to 0.97). The augmented PIM fraction during the winter mixing diluted the quality of the seston (*f*=0.21 to 0.28), bringing a reduction in the AE in the mussels' gut (34-54%). Similarly, other bivalve species increased their AE when they feed on seston of high quality (Cranford, 1995; Hawkins et al., 1996; Iglesias et al., 1996; Wong and Cheung, 2003; Velasco and Navarro, 2003).

Energy expenditure can be estimated by measuring the oxygen consumption and ammonia excretion rates ($VO_2$ and $VNH_4$-N). These metabolic rates were negatively correlated with the chlorophyll and the phytoplankton size-fractions. Nanophytoplankton, the most abundant size-class, explained the highest variation of $VO_2$ (43 %) and $VNH_4$-N (67-70%). This relationship explained why mussels showed the highest metabolic rates (2.67 ml h$^{-1}$ and 26 µg h$^{-1}$) during the summer stratification events. The low chlorophyll levels and the drastic change in water temperature registered during this period probably lead to an imbalance between the energy expended and the energy acquired. Under this conditions, the level of metabolic energy expenditure surpassed the gains of the energy absorbed, resulting in a negative Scope for Growth during the summer stratification events (-34.57 J h$^{-1}$). In contrast, the Scope for Growth showed positive mean values during the spring (16 J h$^{-1}$) and autumn bloom (13.7 J h$^{-1}$), when mussels showed the highest feeding and digestive rates and the energy used in respiration and excretion was low. The lower rates of clearance and absorption obtained under conditions of low food quality during the winter mixing were probably the determinants for the low SFG (1.15 J h$^{-1}$) registered during this period. Multiple regression analyses further corroborated this pattern, as the chlorophyll and $f$ were the factors that explained the highest variation of the SFG (Pérez-Camacho et al., 1995; Wong and Cheung, 2003).

The seasonal fluctuations in the Scope for Growth were coupled with the cycles of storage and expenditure of energetic reserves that occur in bivalves during the annual reproductive cycle. Energy reserves are usually stored during periods of food abundance, when the gonad is at a resting stage, so they can be utilized to meet the energetic requirements for the gonad development during gametogenesis. The separate analysis of the proximate composition of the mantle and the digestive gland revealed distinct patterns of storage and utilization of biochemical reserves, a fact that is probably linked to the distinct physiological functions of each organ. The mantle is the tissue that supports the growth of the shell (Palmer, 1992), the development of the gonad and is the principal storage organ of glycogen (Zandee et al., 1980; Mathieu and Lubet, 1993), a fact that was corroborated by the high average levels found in the mantle compared to the digestive gland (76 and 35 % of total carbohydrates, respectively). On

the other hand, the digestive gland digests and absorbs nutrients, transfers reserves to other tissues during periods of stress and is the principal storage organ of lipids (Zandee et al., 1980; Cartier et al., 2004). Proteins and lipids reserves followed an opposite pattern in the digestive gland in comparison with the mantle, suggesting transference of lipids and protein reserves from the gland to the mantle to support gonadal development during periods of food scarcity (Caers et al., 1999, 2003; Martínez-Pita et al., 2012). The consumption of the glycogen reserves of the mantle during winter suggested that glycogen was being converted to lipids to fuel vitellogenesis (San Juan et al., 1998). The expenditure of lipid and glycogen reserves were followed by a subsequent decline in the Condition Index (18.6 %) and DWt (1569 mg dry weight), which further resulted in a negative Scope for Growth (-1 to 4 J $h^{-1}$) during the winter downwelling period. In contrast, the mantle gained proteins and lipids reserves during the spring bloom and they remained high until summer, following the typical mass spawning period that occurs during late spring (Peteiro et al., 2011; Villalba, 1995). Accordingly, mussels experienced the greatest growth rate (0.05-0.07 g total dry weight day$^{-1}$), a high Condition Index (26%) and Scope for Growth (12-13 J $h^{-1}$) during the spring bloom, when seston loads reached the highest nutritional quality (7 J $l^{-1}$, 94-97% POM). When temperatures rose and chlorophyll levels became scarce during the summer stratification, mussels reduced their energy intake and augmented the metabolic expenditure, leading to a significant decline in the Scope for Growth (-24 to -45 J $h^{-1}$) and the Condition Index (19 %). Short-term imbalances between energy acquisition and expenditure seemed to be compensated by the utilization of protein and lipid reserves storage in the mantle. Mussels eventually restored the glycogen levels in the mantle during the autumn bloom, leading to an enhancement in the Condition Index (31-32 %) and the Scope for Growth (10 to 18 J $h^{-1}$).

The analysis of the fatty acid signature of the mantle and digestive gland of *Mytilus galloprovincialis* further reflected the dynamics of the temporal successions in the plankton community. This was in good agreement with other studies that also verified the potential of fatty acids as trophic markers to identify the different food sources of marine bivalves (Budge

et al., 2001; Alkanani et al., 2007; Shin et al., 2008; Ventrella et al., 2008; Prato et al., 2010; Ezgeta-Balić et al., 2012; Najdek et al., 2013; Zhao et al., 2013; Richoux et al., 2014). Thus, the mantle and the digestive gland showed significantly higher levels of diatom and bacteria FA biomarkers during the upwelling period (spring-summer), while the high abundance of dinoflagellate-derived biomarkers suggested that dinoflagellates constituted the main diet during downwelling (autumn-winter). Short-term changes in the feeding ecology of mussels were reflected more clearly in the digestive gland than in the mantle tissue, probably because the digestive gland is the food processing organ and has a faster turnover rate than the mantle (Shin et al., 2008; Redmond et al., 2010). Both tissues were characterized by a high relative abundance of 16:0, 16:1ω7, 20:5ω3 (EPA) and 22:6ω3 (DHA), which agrees with the composition described for the mantle and the gland of *M. galloprovincialis* and *Perna viridis* (Shin et al., 2008; Ezgeta-Balić et al., 2012). The higher concentrations of EPA and DHA in the mantle and the gland relative to the amount found in the seston suggested a preferential accumulation of these ω3 fatty acids in the mussels' tissues. Given the limited ability of bivalves to elongate and desaturate FA (Albentosa et al., 1996; Alkanani et al., 2007; Fernández-Reiriz et al., 2011; Ventrella et al., 2013), it is highly unlikely that EPA and DHA were biosynthesized from precursor 18:3ω3. In addition, the results showed that the unsaturation degree of the fatty acids of the mussels' tissues increased during the colder seasons (i.e. higher PUFA during autumn-winter) and decreased during the warmer seasons (i.e. higher SAFA and MUFA during spring-summer). Other authors also noticed this pattern in different tissues of bivalve molluscs (Napolitano and Ackman, 1993; Pirini et al., 2007; Ezgeta-Balić et al., 2012). It is thought that the high degree of unsaturation of PUFA confers a low melting point to phospholipids hydrophobic tails and maintains membrane fluidity at colder temperatures (Hall et al., 2002). Furthermore, the results of the multivariate analyses demonstrated that the fatty acid signature of the mussel feces resembled most closely the composition of the seston (23% dissimilarity in composition, SIMPER) than the mantle and the digestive gland (39 and 35 % dissimilarity, respectively, SIMPER). This similarity in composition suggested that mussels recycle a large proportion of the fatty acids contained in the

seston through their biodeposits, so the egested material could be further exploited by other trophic levels in the ecosystem of the Rías.

In this thesis, the integration of the results derived from the physiological energetics, biometrics, proximate composition and fatty acids analyses revealed a different performance for mussels cultured near fish cages in the Ría Ares-Betanzos and the Bay of Fundy.

Mussels cultured near fish cages in Ría Ares-Betanzos showed no significant assimilation of waste fish feed fatty acid markers in their tissues (mantle and digestive gland) or egested material (feces). Considering that the amount and quality of food (chlorophyll-*a*, %POM and proximate composition of the seston) and environmental characteristics of the water (temperature, salinity, oxygen, pH) were comparable among mussel sites, it is not surprising that mussels cultured near and distant from the fish cages showed a similar Scope for Growth, growth rate, meat yield and biochemical composition. The only spatial differences in seston quality owed to short-term resuspension events detected at the raft closest to the fish cages during winter and autumn. During this period, seston showed significantly lower quality, proteins, carbohydrates and lipid content at the raft near the net-pens (38.91 % POM and 12.8%, 5.7% and 6% dry weight, respectively) than at the other raft (60.52 % POM and 20.5%, 8.7%, 10.5% dry weight, respectively). It is highly probable that the stormy conditions typical of this period resulted in an intense resuspension of inorganic benthic sediments that reduced the quality of the seston available for filter-feeders at the raft closest to the fish cages. The fact that resuspension events were more patent at this raft than at the raft distant from the fish cages might be explained by two different reasons. Firstly, this raft was located closer to the coast, at the innermost region of the raft polygon, so it is probable that it was exposed to a higher input of sediments transported by wind or continental runoff. Secondly, the shallower conditions found at this raft might have lead to enhanced current velocities that induced bottom surge stress and resuspension of sediment material during stormy conditions. The enhanced turbidity of the feeding environment was reflected at physiological and biochemical levels in the mussels cultured at the raft closest to the fish cages. The reduction in food quality was coupled with

significant reductions in the absorption efficiency of organic particles in the mussels' gut at the raft near the fish cages (34-66%) compared to mussels cultured in the outermost raft (54-91%). The significantly higher clearance rate measured for mussels cultured near the fish cages seemed to be counteracting the decline in food absorption registered throughout the resuspension events. Bivalves are known to have a high physiological plasticity in their feeding behaviour to obtain a maximum energy gain under the varying concentrations and quality of particulate material suspended in the seawater. This physiological compensation in the feeding rate allowed maintaining a similar SFG compared to mussels cultured at the raft distant from the fish cages. However, it is likely that the reductions in AE determined the temporal decline in the Condition Index and the depletion of proteins and lipid reserves found in the mantle of innermost mussels. Nonetheless, the spatial differences in food quality were short-termed, since the physiological and biochemical performance of mussels cultured at both rafts was very similar outside the resuspension events.

Unlike mussels cultured in Ría Ares-Betanzos, mussels cultured adjacent to the salmon cages in the Bay of Fundy (Canada) were assimilating fish feed fatty acid markers into their tissues and thus, utilizing some fish particulate waste as part of their diet. This was identified by the significantly higher content of feed FA markers 20:1ω9, 18:2ω6, 18:1ω9 and low ω3/ω6 ratio contained in the seston, mussel tissues and feces from the IMTA site compared to the monoculture farm. Nonetheless, the assimilation of fish feed wastes in the Bay of Fundy was not enough to augment the SFG and the amount of biochemical reserves found in IMTA mussels in comparison with monoculture individuals in the Bay of Fundy. In fact, the SFG reflected the spatial differences in food quality found between both sites during tidal-induced resuspension events. Short-term resuspension events significantly reduced POM loads, proteins, carbohydrates and lipids in the seston of the IMTA site (0.5 mg l$^{-1}$, 8.9%, 4.3%, 4.6%, respectively) in comparison with the monoculture site (0.7 mg l$^{-1}$, 18.8%, 8.4%, 14.4%, respectively). Consequently, the significantly lower absorption of organic material in the gut of IMTA mussels during resuspension events lead to a lower SFG (28.71 J h$^{-1}$) than monoculture individuals (38.71 J h$^{-1}$). Furthermore, the similar proximate composition of the mussel tissues

highlighted that IMTA and monoculture mussels were generally exposed to a diet of similar characteristics outside the short-term resuspension events. Thus, even if the assimilation and egestion of fish feed FA markers revealed that mussels were biomitigating some fish feed particles, the comparable proximate composition and the lower SFG indicated that fish feed represented a small part of the mussels' diet. This additional dietary source was not enough to compensate for the short-term reductions in seston quality during resuspension events.

The contrasting results obtained in the Ría Ares-Betanzos and the Bay of Fundy should be discussed considering that both experimental sites represented opposite hypothetical IMTA scenarios. The differences in: i) current speed, ii) seston characteristics and primary production and, iii) husbandry practices of each site, could have played a major role in the different success of the co-cultivation at each site.

i) **Current speed** has been recognized as a key factor for the dispersion and dilution of fish feed waste in integrated aquaculture systems (Troell and Norberg, 1998; Cheshuk et al., 2003; Handå et al., 2012; Cranford et al., 2013). The experimental site in the Ría Ares-Betanzos (Galicia, Spain) has been described as an area with energetic hydrodynamic conditions (Sánchez-Mata et al., 1999; Zuñiga et al., 2014; Piedracoba et al., 2014). Current speed was particularly enhanced at the raft near the fish cages, given its location at the shallower innermost region of the raft polygon. This raft was subjected to fast residual current speeds (average 3.7 cm s$^{-1}$) that reached an annual maximum of 12 cm s$^{-1}$ (Zuñiga et al., 2014; Piedracoba et al., 2014). Consequently, the dilution effect of the current could be a major factor explaining the lack of enhancement of POM and chlorophyll levels in the raft near the fish cages in comparison with the raft moored further away. In addition, seston had a similar proximal composition and fatty acid signature in both rafts. On the other hand, mussels suspended next to the salmon cages in the Bay of Fundy were cultured in a site with lower current speeds (0-5 cm s$^{-1}$; Chang and Page, 2011), that would prevent such a rapid dilution of fish solid and dissolved wastes. Indeed, short-term bulk measurements of seston at the IMTA site showed significantly higher levels of chlorophyll (3.7 µg l$^{-1}$) and fish feed FA biomarkers 20:1ω9 and 18:2ω6 in the seston compared with the monoculture site (2.5 µg l$^{-1}$). The isotopic signature of seston at both sites also proved

to be significantly different. It is probable that ongoing resuspension events at the IMTA site during the study could have masked any enhancement in POM loads coming from the fish cages. The Bay of Fundy is a macro-tidal ecosystem with marked shifts in seston loads over small spatial scales and the levels of POM and chlorophyll detected in this study were always within the range of natural variations (MacDonald et al., 2011; Nelson et al., 2012; Lander et al., 2013). Given that our results are based on a single short-term study we cannot conclude that chlorophyll levels and seston POM were constantly enhanced near the fish cages. Therefore, more intensive sampling series would be required to understand if chlorophyll and POM loads are constantly enhanced at this IMTA site.

ii) Several studies have suggested that mussels grown in the vicinity of fish farms might only consume feed waste as an alternative food source when **natural seston loads are scarce** during winter or autumn (Stirling and Okumus, 1995; Troell et al., 2003; Cheshuk et al., 2003; Gao et al., 2006; Handå et al., 2012a; Both et al., 2012; Lander et al., 2013). However, the study carried out in Ría Ares-Betanzos showed no evidence of seasonal-driven fluctuations in the incorporation of feed fatty acid markers by seston or the filter-feeders. Thus, it is probable that mussels were not restricted by a limited supply of seston or chlorophyll (annual range 30 to 60 % POM and 0.38 to 1 $\mu$g l$^{-1}$), as seen by the high growth rates and positive Scope for Growth measured during the entire experimental period (annual range 0.05 to 0.06 g day$^{-1}$ total dry weight and -1.79 to 10.75 J h$^{-1}$), excepting for the negative SFG found during transient summer stratification events.

iii) The **differences in husbandry practices** between Spain and Canada could be the last major factor explaining the different performance obtained for mussels cultured near fish cages in both sites. Mussels in Spain were cultured at a greater distance (170m) from the fish cages than in the Bay of Fundy, where mussels were reared on rafts immediately adjacent to salmon cages. Thus, it is probable that there was a dilution effect of fish feed waste particles with increasing distance from the net-pens. Consequently, mussels cultured near the fish cages in Ría Ares-Betanzos would potentially have a lower exposure to feed particles in comparison with mussels reared in the Bay of Fundy IMTA. This has also been suggested by other open-

water IMTA studies, in which most fish-derived POM was detected within 0 to 60 m from the fish cages (Cheshuk et al., 2003; Lander et al., 2013). It is worth highlighting that the position of the mussel rafts in the Bay of Fundy tried to obey a hypothetical optimal design for fish waste exploitation by shellfish growing in open-water IMTA, while the position of the rafts in Galicia could not be modified as it is strictly regulated through coastal management plans. Furthermore, the differences in fish and shellfish stocking density among both sites could also be a factor contributing to the lack of assimilation of fish feed waste by mussels reared in Ría Ares-Betanzos. Fish effluents in the latter site were spread across a larger biomass of bivalves ($1440 \times 10^3$ to $4710 \times 10^3$ mussels raft$^{-1}$) than in the Bay of Fundy (~1,000 mussels sock$^{-1}$). Moreover, the amount of fish feed particles exiting the net-pens was probably lower in the red sea bream farm of the Ría Ares-Betanzos (450 annual tonnes of sea bream; 70 tonnes during each sampling occasion) than in the salmon farm of the Bay of Fundy (15 kg m$^{-3}$ salmon in net-pens of 8478 m$^3$).

The interaction of the specific hydrodynamic characteristics, seston loads and husbandry practices of the Spanish site reduced the possibility of detecting an enhancement in mussels' SFG, meat yield, biochemical reserves or assimilation of fish feed fatty acid markers compared to those mussels growing in the Canadian IMTA site.

From all the above, this thesis **<u>concluded</u>** that:

**1.-**Bivalves cultured in suspension in coastal areas are subjected to long-term and short-term variations in the feeding environment. Long-term fluctuations in seston quality and quantity are driven by the succession of upwelling and downwelling episodes in the Galician Rías. Seston showed the highest nutritional quality during the spring and autumn bloom, while summer and winter were the periods with the lowest chlorophyll levels and nutritional quality, respectively. Nanophytoplankton was the dominant size-fraction during all seasons. Short-term changes in seston loads can result from the resuspension of bottom material during storms or

tidal cycles and can generate differences in food quality between mussel culture units located within a small geographical scale.

**2.-**Seasonal variations in the Scope for Growth, growth, Condition Index, proximate composition and fatty acid signature of the mussels were closely linked with the natural fluctuations in the quality and composition of the diet.

**3.-**The seasonal variability in absorption efficiency was best explained by the food quality ($f$). Food absorption increased with $f$ during the spring bloom and reached minimum levels with the lowest food quality found during winter mixing. Food absorption decreased with the lowest food quality found during winter and autumn resuspension events at the innermost raft. Similarly, reductions in food quality during tidal-induced resuspension events also resulted in a lower absorption efficiency.

**4.-**Although the clearance rate and the organic ingestion rate varied as a function of the total chlorophyll-$a$, nanophytoplankton (2-20 µm) and microphytoplankton (>20-50 µm) explained a higher variability than picophytoplankton (0.2-2 µm), suggesting that the former size-fractions were preferentially cleared and ingested by mussels.

**5.-**The respiration and ammonia excretion rates showed a negative correlation with the amount of chlorophyll, as demonstrated by the maximum metabolic rates found during the summer stratification events, when chlorophyll levels were significantly depleted.

**6.-**Seasonal variations in Scope for Growth were best explained by fluctuations in chlorophyll levels and $f$ during upwelling-downwelling episodes. The high temperature of the water and the low chlorophyll levels detected during the summer stratification events resulted in reduced energy intake and significantly higher metabolic expenditure. This was translated in a negative Scope for Growth. In contrast, the high nutritional quality of the seston during the spring and autumn bloom increased the energy intake and reduced the metabolic expenditure, resulting in an augmented Scope for Growth.

**7.-**Temporal increases in turbidity during storm-induced resuspension events did not seem to significantly decrease mussels' Scope for Growth, growth rates and proximate composition over a long time scale. The significant reduction in the absorption efficiency with

reduced food quality seemed to be compensated by an enhancement in the clearance rate of food particles in the ecosystems of the Ría Ares-Betanzos and the Bay of Fundy.

**8.-**The analysis of the proximate composition of two separated organs revealed a distinct pattern in the storage and utilization of reserves. Proteins and lipids are probably transferred from the digestive gland to the mantle tissue for gamete production during winter downwelling and stored in the digestive gland during the summer upwelling. The glycogen reserves of the mantle are probably converted to lipids to support vitellogenesis during winter.

**9.-**The temporal variations in fatty acid composition of the seston were reflected in the fatty acid profile of the mussels' organs and feces. The dominance of diatom and bacteria-derived fatty acid markers in the mantle and digestive gland of the mussels during the upwelling period suggested that they constituted the predominant food sources during spring-summer. In contrast, dinoflagellates were the most abundant food source during the downwelling period.

**10.-**The comparable feeding environment (TPM, PIM, POM, chlorophyll, proximate composition of the seston) and physico-chemical characteristics found at the mussel culture units located near and distant from the fish cages resulted in a similar Scope for Growth, growth rates, Condition Index and proximate composition for mussels cultured at both sites in Spain and Canada.

**11.-** There was no evidence of any seasonal or spatial incorporation of fish feed FA markers in the mantle, digestive gland or feces of mussels cultured near fish cages in Spain. However, there was a significant assimilation and egestion of fish feed fatty acid markers by mussels cultured in the IMTA site in Canada. This dietary enhancement was probably small and did not result in an augmented Scope for Growth, proteins, carbohydrates or lipid reserves. Fish feed fatty acid markers are powerful tracers for studying the assimilation of fish feed uneaten particles by mussels cultured in proximity to fish cages over a short-term study. In contrast, short-term measurements of the physiological rates and proximate composition of mussel tissues provided limited information of any dietary enhancement derived from the assimilation of fish feed waste in IMTA sites.

**12.-** A meaningful assimilation of fish feed particles and a subsequent enhancement in bivalve growth might only happen when bivalves are cultured very close to the fish cages (<170 m) in ecosystems with prolonged periods of low food quality (<50% organic content) and moderate current speeds (0-5 cm s$^{-1}$).

SUPPLEMENTARY MATERIAL

TABLES CHAPTER 5

FATTY ACIDS AS TRACERS OF TROPHIC INTERACTIONS BETWEEN SESTON, MUSSELS AND BIODEPOSITS IN A COASTAL EMBAYMENT OF MUSSEL RAFTS IN THE PROXIMITY OF FISH CAGES

| FAME | Summer 2010 | | Autumn 2010 | | Winter 2011 | | Spring 2011 | | Autumn 2011 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 |
| C 14:0 | 8.50 ± 3.25 | 7.72 ± 0.76 | 8.09 ± 1.84 | 8.43 ± 0.71 | 10.13 ± 0.28 | 9.69 ± 2.08 | 9.73 ± 0.37 | 11.44 ± 0.33 | 8.11 ± 1.45 | 5.85 ± 0.40 |
| C 15:0 | 3.01 ± 0.73 | 3.09 ± 0.23 | 3.37 ± 1.12 | 3.65 ± 0.37 | 3.74 ± 0.20 | 3.25 ± 0.60 | 2.65 ± 0.88 | 2.58 ± 0.41 | 3.28 ± 0.94 | 2.68 ± 0.60 |
| C 16:0 | 23.21 ± 2.13 | 22.95 ± 0.85 | 26.2 ± 3.32 | 27.88 ± 2.32 | 30.67 ± 1.20 | 26.89 ± 3.38 | 25.91 ± 2.16 | 28.25 ± 1.37 | 28.11 ± 1.76 | 26.55 ± 6.99 |
| C 16:1ω9 | 6.37 ± 0.84 | 7.46 ± 0.76 | 6.22 ± 1.18 | 8.04 ± 2.05 | 8.2 ± 0.66 | 8.19 ± 2.15 | 4.67 ± 3.11 | 3.74 ± 1.37 | 9.91 ± 3.21 | 5.55 ± 1.81 |
| C 16:1ω7 | 9.59 ± 1.63 | 8.42 ± 1.29 | 6.56 ± 1.73 | 7.48 ± 1.70 | 8.07 ± 0.69 | 7.33 ± 1.65 | 9.32 ± 1.40 | 11.65 ± 2.39 | 7.19 ± 1.71 | 7.25 ± 1.95 |
| C 17:0 | 1.28 ± 0.44 | 1.38 ± 0.21 | 1.23 ± 0.37 | 1.56 ± 0.20 | 2.21 ± 0.08 | 2.06 ± 0.26 | 1.62 ± 0.36 | 1.71 ± 0.45 | 1.95 ± 0.37 | 1.67 ± 0.45 |
| C DMA 18:0 | 1.15 ± 0.25 | 0.98 ± 0.31 | 1.02 ± 0.28 | 1.35 ± 0.14 | 1.26 ± 0.04 | 1.32 ± 0.14 | 1.14 ± 0.35 | 0.98 ± 0.29 | 1.43 ± 0.32 | 1.05 ± 0.27 |
| C 18:0 | 7.64 ± 0.89 | 9.62 ± 0.80 | 11.15 ± 3.32 | 11.33 ± 6.10 | 8.58 ± 2.43 | 9.98 ± 3.47 | 13.64 ± 10.08 | 11.27 ± 6.21 | 4.15 ± 2.81 | 10.23 ± 3.78 |
| C 18:1ω9 | 14.7 ± 12.16 | 9.29 ± 3.20 | 14.77 ± 15.70 | 10.49 ± 7.45 | 6.16 ± 3.35 | 11.77 ± 5.75 | 7.62 ± 0.10 | 5.60 ± 1.74 | 12.64 ± 5.36 | 7.25 ± 1.99 |
| C 18:1ω7 | 6.65 ± 1.57 | 5.85 ± 1.39 | 5.07 ± 1.29 | 5.30 ± 1.60 | 5.37 ± 1.31 | 3.22 ± 0.55 | 6.41 ± 1.61 | 7.08 ± 1.64 | 4.80 ± 1.23 | 5.66 ± 1.66 |
| C 18:2ω6 | 3.19 ± 0.90 | 2.57 ± 0.18 | 3.32 ± 1.50 | 2.90 ± 0.69 | 2.35 ± 1.36 | 3.72 ± 1.87 | 3.34 ± 2.18 | 1.46 ± 1.03 | 4.91 ± 3.24 | 3.32 ± 0.94 |
| C 18:3ω6 | - | - | - | - | - | - | - | - | - | - |
| C 18:3ω3 | 0.59 ± 0.05 | 0.68 ± 0.14 | 0.69 ± 0.06 | 0.71 ± 0.12 | 1.01 ± 0.17 | 0.89 ± 0.20 | 0.69 ± 0.06 | 1.00 ± 0.18 | 0.82 ± 0.15 | 0.41 ± 0.05 |
| C 18:4ω3 | 0.68 ± 0.03 | 0.79 ± 0.65 | 0.92 ± 0.32 | 0.75 ± 0.22 | 2.14 ± 0.32 | 1.73 ± 0.20 | 1.04 ± 0.04 | 1.74 ± 0.29 | 1.07 ± 0.34 | 0.6 ± 0.14 |
| C 20:1ω11 | - | - | 0.13 ± 0.22 | - | - | 0.27 ± 0.46 | 0.60 ± 0.14 | 0.36 ± 0.32 | 0.22 ± 0.39 | - |
| C 20:1ω9 | 0.22 ± 0.32 | 0.15 ± 0.25 | 0.10 ± 0.17 | - | 1.03 ± 0.29 | 1.51 ± 0.53 | 1.74 ± 0.38 | 1.37 ± 0.39 | 3.34 ± 1.15 | 2.16 ± 0.11 |
| C 20:1ω7 | 2.47 ± 1.24 | 1.74 ± 0.86 | 1.32 ± 0.40 | 1.25 ± 0.52 | - | - | - | - | 0.08 ± 0.15 | 0.28 ± 0.03 |
| C 20:2NMI1 | - | - | - | - | - | - | - | - | 0.40 ± 0.16 | 0.26 ± 0.12 |
| C 20:2NMI2 | - | - | - | - | - | - | - | - | - | - |
| C 20:2ω6 | - | - | - | - | - | - | - | - | - | 0.10 ± 0.17 |
| C 20:4ω6 | 5.68 ± 1.32 | 13.5 ± 8.91 | 5.62 ± 1.82 | 5.73 ± 2.26 | 3.59 ± 0.72 | 3.30 ± 0.66 | 4.54 ± 2.22 | 2.70 ± 2.45 | 1.21 ± 0.29 | 1.64 ± 0.50 |
| C 20:4ω3 | - | - | - | - | - | - | - | - | - | - |
| C 20:5ω3 | 2.44 ± 1.02 | 1.82 ± 0.39 | 1.50 ± 0.53 | 1.26 ± 0.04 | 1.82 ± 0.31 | 2.23 ± 0.27 | 1.71 ± 0.17 | 2.56 ± 0.69 | 1.36 ± 0.28 | 1.02 ± 0.41 |
| C 22:0 | 0.55 ± 0.28 | 0.56 ± 0.23 | 1.20 ± 0.44 | 0.87 ± 0.29 | 1.47 ± 0.09 | 0.69 ± 0.08 | 1.13 ± 0.04 | 1.83 ± 0.20 | 1.40 ± 0.29 | 0.88 ± 0.19 |
| C 22:2NMID1 | - | - | - | - | - | - | - | - | - | 0.07 ± 0.12 |
| C 22:2NMID2 | - | - | - | - | - | - | 0.48 ± 0.06 | - | 0.40 ± 0.02 | 12.46 ± 0.88 |
| C 22:3 NMIT | - | - | - | - | 0.24 ± 0.41 | 0.17 ± 0.29 | - | - | 0.14 ± 0.24 | 0.16 ± 0.27 |
| C 22:4ω6 | - | - | - | - | - | - | - | - | - | - |
| C 22:5ω6 | - | - | - | - | - | - | - | - | - | - |
| C 22:5ω3 | 0.56 ± 0.30 | 0.38 ± 0.33 | 0.16 ± 0.28 | - | 0.91 ± 0.11 | 0.93 ± 0.22 | 0.56 ± 0.02 | 0.38 ± 0.34 | 0.96 ± 0.24 | 0.54 ± 0.13 |
| C 22:6ω3 | 1.51 ± 0.06 | 1.05 ± 0.21 | 1.35 ± 0.48 | 1.02 ± 0.25 | 1.06 ± 0.18 | 0.87 ± 0.07 | 1.43 ± 0.07 | 2.30 ± 0.44 | 2.10 ± 0.69 | 2.38 ± 0.93 |

Table S1. Spatial and temporal variations of the fatty acid (FA) composition of the seston (n = 30). The content is expressed as relative percentage of total FA (mean ± SD). The – indicates the FA was below the detection limit.

| FAME | Summer 2010 | | Autumn 2010 | | Winter 2011 | | Spring 2011 | | Autumn 2011 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 |
| SAFA | 44.19 ± 7.72 | 45.32 ± 2.47 | 51.24 ± 10.17 | 53.72 ± 8.41 | 56.8 ± 3.59 | 52.56 ± 6.18 | 54.68 ± 6.35 | 57.08 ± 6.30 | 47.00 ± 1.89 | 47.85 ± 11.92 |
| MUFA | 40.00 ± 7.21 | 32.91 ± 5.31 | 34.17 ± 11.56 | 32.55 ± 9.54 | 28.82 ± 2.63 | 32.28 ± 5.41 | 30.38 ± 5.98 | 29.80 ± 6.33 | 38.19 ± 0.70 | 28.15 ± 7.26 |
| PUFA | 14.65 ± 0.76 | 20.79 ± 7.87 | 13.57 ± 2.08 | 12.37 ± 1.30 | 13.12 ± 0.98 | 13.84 ± 0.95 | 13.80± 0.02 | 12.14 ± 1.06 | 13.38 ± 2.72 | 22.95 ± 19.43 |
| DMA | 1.15 ± 0.25 | 0.98 ± 0.31 | 1.02 ± 0.28 | 1.35 ± 0.14 | 1.26 ± 0.04 | 1.32 ± 0.14 | 1.14 ± 0.35 | 0.98 ± 0.29 | 1.43 ± 0.32 | 1.05 ± 0.27 |
| ω3 | 5.78 ± 1.46 | 4.72 ± 1.58 | 4.63 ± 1.47 | 3.74 ± 0.40 | 6.94 ± 0.95 | 6.65 ± 0.87 | 5.44 ± 0.12 | 7.98 ± 1.29 | 6.32 ± 0.19 | 4.94 ± 1.03 |
| ω6 | 8.87 ± 2.22 | 16.07 ± 8.78 | 8.94 ± 1.37 | 8.63 ± 1.64 | 5.94 ± 0.86 | 7.01 ± 1.37 | 7.88 ± 0.04 | 4.17 ± 1.43 | 6.12 ± 3.15 | 5.06 ± 0.87 |
| ω7 | 18.71 ± 4.43 | 16.01 ± 2.62 | 12.95 ± 3.36 | 14.03 ± 3.18 | 13.44 ± 0.63 | 10.55 ± 2.19 | 15.73 ± 3.01 | 18.73 ± 4.02 | 12.07 ± 2.81 | 13.19 ± 3.64 |
| ω9 | 21.29 ± 11.64 | 16.90 ± 2.88 | 21.09 ± 14.78 | 18.52 ± 8.07 | 15.39 ± 2.98 | 21.47 ± 4.81 | 14.04 ± 2.83 | 10.71 ± 2.73 | 25.89 ± 2.49 | 14.96 ± 3.64 |
| ω11 | - | - | 0.13 ± 0.22 | - | - | 0.27 ± 0.46 | 0.60 ± 0.14 | 0.36 ± 0.32 | 0.22 ± 0.39 | - |
| PUFA ω3 | 5.78 ± 1.46 | 4.72 ± 1.58 | 4.63 ± 1.47 | 3.74 ± 0.40 | 6.94 ± 0.95 | 6.65 ± 0.87 | 5.44 ± 0.12 | 7.98 ± 1.29 | 6.32 ± 0.19 | 4.94 ± 1.03 |
| ω3/ω6 | 0.69 ± 0.34 | 0.40 ± 0.34 | 0.53 ± 0.19 | 0.45 ± 0.14 | 1.19 ± 0.27 | 0.98 ± 0.28 | 0.69 ± 0.02 | 2.15 ± 1.05 | 1.20 ± 0.49 | 1.00 ± 0.31 |
| NMI | - | - | - | - | 0.24 ± 0.41 | 0.17 ± 0.29 | 0.48 ± 0.06 | - | 0.94 ± 0.38 | 12.95 ± 0.57 |
| NMID | - | - | - | - | - | - | 0.48 ± 0.06 | - | 0.80 ± 0.18 | 12.79 ± 0.70 |
| NMIT | - | - | - | - | 0.24 ± 0.41 | 0.17 ± 0.29 | - | - | 0.14 ± 0.24 | 0.16 ± 0.27 |

Table S2. Spatial and temporal variations of the fatty acid (FA) composition of the seston (n = 30). The content is expressed as relative percentage of total FA (mean ± SD). The – indicates the FA was below the detection limit. SAFA = saturated FA, MUFA = monounsaturated FA, PUFA = polyunsaturated FA, DMA= dimethyl acetals FA, PUFA ratio for the ω3 and ω6 series, NMI= non-methylene-interrupted FA, NMID= NMI dienoic FA, NMIT= NMI trienoic FA.

| FAME | Summer 2010 | | Autumn 2010 | | Winter 2011 | | Spring 2011 | | Autumn 2011 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 |
| C 14:0 | 3.12 ± 0.06 | 2.60 ± 0.59 | 2.32 ± 0.26 | 1.58 ± 0.41 | 1.06 ± 0.95 | 1.36 ± 0.97 | 2.312 ± 1.18 | 3.61 ± 0.88 | 1.40 ± 0.72 | 1.66 ± 0.46 |
| C 15:0 | 0.56 ± 0.02 | 0.55 ± 0.03 | 0.52 ± 0.06 | 0.62 ± 0.08 | 0.40 ± 0.16 | 0.43 ± 0.14 | 0.37 ± 0.06 | 0.40 ± 0.02 | 0.48 ± 0.06 | 0.54 ± 0.03 |
| C 16:0 | 19.27 ± 0.40 | 18.19 ± 0.65 | 20.71 ± 0.26 | 20.06 ± 0.75 | 17.09 ± 1.17 | 16.98 ± 1.24 | 16.65 ± 0.32 | 17.28 ± 0.53 | 19.54 ± 2.07 | 20.89 ± 0.86 |
| C 16:1ω9 | 0.23 ± 0.02 | 0.21 ± 0.04 | 0.19 ± 0.05 | 0.18 ± 0.02 | 0.35 ± 0.12 | 0.14 ± 0.12 | 0.16 ± 0.01 | 0.20 ± 0.03 | 0.21 ± 0.01 | 0.24 ± 0.08 |
| C 16:1ω7 | 8.36 ± 0.61 | 7.61 ± 1.04 | 5.50 ± 0.39 | 4.58 ± 0.79 | 2.72 ± 1.56 | 5.42 ± 3.69 | 4.97 ± 2.34 | 8.28 ± 1.94 | 3.74 ± 1.41 | 4.69 ± 1.19 |
| C 17:0 | 0.62 ± 0.08 | 0.70 ± 0.08 | 0.61 ± 0.02 | 0.62 ± 0.03 | 1.06 ± 0.22 | 0.90 ± 0.20 | 0.64 ± 0.42 | 0.72 ± 0.16 | 0.73 ± 0.20 | 0.57 ± 0.05 |
| C DMA 18:0 | 6.28 ± 0.42 | 6.90 ± 0.64 | 5.18 ± 0.36 | 5.31 ± 0.59 | 7.70 ± 1.53 | 6.97 ± 1.82 | 7.76 ± 0.72 | 6.65 ± 0.57 | 5.62 ± 1.04 | 5.14 ± 0.57 |
| C 18:0 | 3.99 ± 0.13 | 4.24 ± 0.54 | 4.45 ± 0.48 | 4.11 ± 0.48 | 3.50 ± 2.44 | 4.58 ± 1.79 | 5.09 ± 1.15 | 3.80 ± 0.94 | 5.44 ± 2.01 | 3.77 ± 0.20 |
| C 18:1ω9 | 1.08 ± 0.12 | 1.03 ± 0.10 | 1.69 ± 0.10 | 1.31 ± 0.10 | 0.88 ± 0.35 | 1.72 ± 1.03 | 0.73 ± 0.16 | 1.06 ± 0.12 | 1.77 ± 0.14 | 1.74 ± 0.65 |
| C 18:1ω7 | 2.76 ± 0.07 | 2.64 ± 0.11 | 1.99 ± 0.07 | 2.05 ± 0.10 | 1.48 ± 0.18 | 1.73 ± 0.15 | 3.30 ± 0.08 | 2.93 ± 0.15 | 1.69 ± 0.12 | 1.93 ± 0.07 |
| C 18:2ω6 | 1.10 ± 0.03 | 1.20 ± 0.05 | 1.66 ± 0.10 | 1.96 ± 0.09 | 1.07 ± 0.34 | 1.18 ± 0.29 | 0.85 ± 0.05 | 0.85 ± 0.05 | 2.03 ± 0.67 | 2.11 ± 0.19 |
| C 18:3ω6 | 0.62 ± 0.07 | 0.59 ± 0.08 | 0.42 ± 0.07 | 0.39 ± 0.05 | 0.92 ± 0.25 | 0.79 ± 0.27 | 1.08 ± 0.24 | 0.78 ± 0.16 | 0.48 ± 0.15 | 0.37 ± 0.04 |
| C 18:3ω3 | 0.79 ± 0.08 | 0.64 ± 0.09 | 0.88 ± 0.06 | 0.60 ± 0.11 | 0.71 ± 0.29 | 0.93 ± 0.33 | 0.59 ± 0.11 | 0.76 ± 0.04 | 0.83 ± 0.15 | 0.82 ± 0.05 |
| C 18:4ω3 | 2.07 ± 0.22 | 1.55 ± 0.48 | 2.50 ± 0.56 | 1.73 ± 0.46 | 1.40 ± 0.99 | 1.94 ± 1.14 | 1.42 ± 0.62 | 2.55 ± 0.33 | 1.87 ± 0.95 | 1.83 ± 0.22 |
| C 20:1ω11 | 1.11 ± 0.04 | 1.08 ± 0.17 | 1.04 ± 0.27 | 1.02 ± 0.09 | 0.65 ± 0.30 | 0.82 ± 0.38 | 0.84 ± 0.27 | 0.92 ± 0.13 | 0.94 ± 0.33 | 0.94 ± 0.12 |
| C 20:1ω9 | 1.72 ± 0.04 | 1.78 ± 0.21 | 1.91 ± 0.23 | 1.98 ± 0.14 | 2.61 ± 0.33 | 2.78 ± 0.34 | 1.97 ± 0.39 | 1.54 ± 0.26 | 2.27 ± 0.38 | 2.09 ± 0.18 |
| C 20:1ω7 | 1.12 ± 0.12 | 0.94 ± 0.08 | 0.88 ± 0.14 | 0.86 ± 0.12 | 0.93 ± 0.04 | 0.98 ± 0.02 | 1.44 ± 0.10 | 1.60 ± 0.14 | 0.91 ± 0.09 | 0.85 ± 0.13 |
| C 20:2NMI1 | 1.43 ± 0.17 | 1.68 ± 0.33 | 1.53 ± 0.27 | 1.69 ± 0.17 | 1.06 ± 0.32 | 1.58 ± 0.59 | 0.89 ± 0.25 | 1.28 ± 0.17 | 1.58 ± 0.40 | 1.77 ± 0.16 |
| C 20:2NMI2 | 0.52 ± 0.07 | 0.67 ± 0.15 | 0.31 ± 0.02 | 0.45 ± 0.06 | 0.27 ± 0.04 | 0.39 ± 0.09 | 0.33 ± 0.07 | 0.39 ± 0.04 | 0.41 ± 0.07 | 0.42 ± 0.05 |
| C 20:2ω6 | 0.49 ± 0.04 | 0.50 ± 0.03 | 0.80 ± 0.05 | 0.81 ± 0.09 | 0.66 ± 0.08 | 0.78 ± 0.12 | 0.53 ± 0.02 | 0.58 ± 0.01 | 0.81 ± 0.14 | 0.85 ± 0.06 |
| C 20:4ω6 | 2.44 ± 0.22 | 2.74 ± 0.22 | 1.38 ± 0.08 | 1.55 ± 0.10 | 2 .00 ± 0.32 | 1.98 ± 0.29 | 2.16 ± 0.25 | 1.62 ± 0.16 | 1.36 ± 0.27 | 1.49 ± 0.12 |
| C 20:4ω3 | 0.32 ± 0.05 | 0.22 ± 0.44 | 0.27 ± 0.02 | 0.13 ± 0.12 | 0.12 ± 0.21 | 0.26 ± 0.23 | 0.23 ± 0.20 | 0.55 ± 0.08 | 0.22 ± 0.08 | 0.17 ± 0.00 |
| C 20:5ω3 | 17.86 ± 0.68 | 16.75 ± 1.31 | 14.55 ± 0.63 | 14.50 ±0.46 | 16.34 ± 1.57 | 14.07 ± 1.60 | 21.28 ± 0.93 | 21.90 ± 0.36 | 14.79 ± 0.47 | 15.06 ± 0.31 |
| C 22:0 | - | - | - | - | - | - | - | - | 0.37 ± 0.45 | 0.08 ± 0.01 |
| C 22:2NMID1 | 0.24 ± 0.05 | 0.24 ± 0.04 | 0.39 ± 0.15 | 0.32 ± 0.05 | 0.12 ± 0.20 | 0.31 ± 0.28 | 0.13 ± 0.11 | 0.19 ± 0.01 | 0.36 ± 0.11 | 0.37 ± 0.13 |
| C 22:2NMID2 | 2.52 ± 0.32 | 2.48 ± 0.38 | 1.51 ± 0.40 | 1.67 ± 0.14 | 1.17 ± 0.47 | 1.53 ± 0.57 | 1.94 ± 0.57 | 2.21 ± 0.13 | 1.22 ± 0.36 | 1.44 ± 0.21 |
| C 22:3 NMIT | 0.45 ± 0.08 | 0.47 ± 0.08 | 0.47 ± 0.14 | 0.59 ± 0.09 | 0.46 ± 0.19 | 0.56 ± 0.17 | 0.30 ± 0.04 | 0.27 ± 0.04 | 0.58 ± 0.15 | 0.67 ± 0.06 |
| C 22:4ω6 | 0.77 ± 0.01 | 0.75 ± 0.04 | 0.54 ± 0.03 | 0.48 ± 0.04 | 0.64 ± 0.11 | 0.7 ± 0.06 | 0.99 ± 0.01 | 1.06 ± 0.04 | 0.58 ± 0.12 | 0.66 ± 0.01 |
| C 22:5ω6 | 0.51 ± 0.05 | 0.54 ± 0.04 | 0.41 ± 0.05 | 0.45 ± 0.02 | 0.43 ± 0.05 | 0.45 ± 0.10 | 0.70 ± 0.08 | 0.54 ± 0.03 | 0.24 ± 0.04 | 0.25 ± 0.03 |
| C 22:5ω3 | 1.43 ± 0.06 | 1.26 ± 0.03 | 1.27 ± 0.02 | 1.34 ± 0.05 | 1.61 ± 0.05 | 1.39 ± 0.17 | 2.10 ± 0.16 | 1.87 ± 0.07 | 1.56 ± 0.05 | 1.38 ± 0.01 |
| C 22:6ω3 | 16.16 ± 1.33 | 19.24 ± 1.60 | 24.12 ± 1.46 | 27.05 ± 1.73 | 30.6 ± 3.94 | 26.35 ± 4.98 | 18.14 ± 2.77 | 13.48 ± 2.05 | 25.98 ± 1.98 | 25.21 ± 2.73 |

Table S3. Spatial and temporal variations of the fatty acid (FA) composition of the mantle tissue (n = 30). The content is expressed as relative percentage of total FA (mean ± SD). The – indicates the FA was below the detection limit.

| FAME | Summer 2010 | | Autumn 2010 | | Winter 2011 | | Spring 2011 | | Autumn 2011 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 |
| SAFA | 27.58 ± 0.47 | 26.29 ± 0.66 | 28.61 ± 0.31 | 26.99 ± 0.68 | 23.11 ± 2.36 | 24.25 ± 0.38 | 25.08 ± 0.71 | 25.82 ± 0.34 | 27.95 ± 0.78 | 27.52 ± 1.11 |
| MUFA | 16.37 ± 0.72 | 15.29 ± 1.07 | 13.20 ± 0.33 | 12.00 ± 1.00 | 9.62 ± 1.88 | 13.59 ± 5.04 | 13.45 ± 2.25 | 16.57 ± 1.73 | 11.52 ± 1.36 | 12.49 ± 1.80 |
| PUFA | 49.76 ± 0.82 | 51.53 ± 1.35 | 53.01 ± 0.25 | 55.7 ± 1.06 | 59.57 ± 2.56 | 55.19 ± 3.62 | 53.71 ± 2.27 | 50.95 ± 1.50 | 54.92 ± 1.22 | 54.86 ± 2.34 |
| DMA | 6.28 ± 0.42 | 6.90 ± 0.64 | 5.18 ± 0.36 | 5.31 ± 0.59 | 7.70 ± 1.53 | 6.97 ± 1.82 | 7.76 ± 0.72 | 6.65 ± 0.57 | 5.62 ± 1.04 | 5.14 ± 0.57 |
| ω3 | 38.64 ± 0.85 | 39.66 ± 2.18 | 43.59 ± 1.02 | 45.35 ± 1.49 | 50.78 ± 3.66 | 44.95 ± 5.09 | 43.78 ± 2.75 | 41.13 ± 1.39 | 45.25 ± 1.30 | 44.46 ± 2.77 |
| ω6 | 5.95 ± 0.29 | 6.32 ± 0.27 | 5.20 ± 0.11 | 5.64 ± 0.16 | 5.71 ± 0.16 | 5.87 ± 0.33 | 6.33 ± 0.55 | 5.46 ± 0.27 | 5.51 ± 0.86 | 5.73 ± 0.06 |
| ω7 | 12.24 ± 0.65 | 11.19 ± 0.93 | 8.37 ± 0.22 | 7.50 ± 0.92 | 5.13 ± 1.70 | 8.13 ± 3.81 | 9.73 ± 2.21 | 12.83 ± 1.76 | 6.34 ± 1.37 | 7.47 ± 1.13 |
| ω9 | 3.02 ± 0.10 | 3.03 ± 0.30 | 3.80 ± 0.15 | 3.48 ± 0.08 | 3.84 ± 0.10 | 4.63 ± 0.87 | 2.88 ± 0.24 | 2.81 ± 0.16 | 4.24 ± 0.51 | 4.08 ± 0.55 |
| ω11 | 1.11 ± 0.04 | 1.08 ± 0.17 | 1.04 ± 0.27 | 1.02 ± 0.09 | 0.65 ± 0.30 | 0.82 ± 0.38 | 0.84 ± 0.27 | 0.93 ± 0.13 | 0.94 ± 0.33 | 0.94 ± 0.12 |
| PUFA ω3 | 38.64 ± 0.85 | 39.66 ± 2.18 | 43.59 ± 1.02 | 45.35 ±1.49 | 50.78 ± 3.66 | 44.95 ± 5.09 | 43.78 ± 2.75 | 41.13 ± 1.39 | 45.25 ± 1.30 | 44.46 ± 2.77 |
| ω3/ω6 | 6.50 ± 0.35 | 6.28 ± 0.51 | 8.38 ± 0.19 | 8.04 ± 0.43 | 8.89 ± 0.59 | 7.65 ± 0.68 | 6.93 ± 0.21 | 7.54 ± 0.12 | 8.32 ± 1.00 | 7.75 ± 0.40 |
| NMI | 5.16 ± 0.65 | 5.54 ± 0.88 | 4.22 ± 0.95 | 4.71 ± 0.46 | 3.07 ± 1.20 | 4.37 ± 1.69 | 3.60 ± 1.02 | 4.37 ± 0.25 | 4.15 ± 1.07 | 4.66 ± 0.50 |
| NMID | 4.72 ± 0.57 | 5.07 ± 0.81 | 3.74 ± 0.81 | 4.13 ± 0.37 | 2.61 ± 1.02 | 3.80 ± 1.53 | 3.30 ± 0.99 | 4.09 ± 0.27 | 3.57 ± 0.92 | 3.99 ± 0.44 |
| NMIT | 0.45 ± 0.08 | 0.47 ± 0.07 | 0.47 ± 0.14 | 0.59 ± 0.09 | 0.46 ± 0.19 | 0.56 ± 0.17 | 0.30 ± 0.04 | 0.28 ± 0.04 | 0.58 ± 0.15 | 0.67 ± 0.06 |

Table S4. Spatial and temporal variations of the fatty acid (FA) composition of the mantle tissue (n = 30). The content is expressed as relative percentage of total FA (mean ± SD).  The – indicates the FA was below the detection limit. SAFA = saturated FA, MUFA = monounsaturated FA, PUFA = polyunsaturated FA, DMA= dimethyl acetals FA, PUFA ratio for the ω3 and ω6 series, NMI= non-methylene-interrupted FA, NMID= NMI dienoic FA, NMIT= NMI trienoic FA.

| FAME | Summer 2010 | | Autumn 2010 | | Winter 2011 | | Spring 2011 | | Autumn 2011 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 |
| C 14:0 | 7.70 ± 0.31 | 7.17 ± 0.63 | 4.06 ± 0.47 | 3.61 ± 0.50 | 1.79 ± 0.46 | 1.60 ± 0.55 | 8.07 ± 1.10 | 5.52 ± 0.46 | 2.71 ± 0.48 | 3.21 ± 0.12 |
| C 15:0 | 0.60 ± 0.04 | 0.58 ± 0.11 | 0.53 ± 0.01 | 0.56 ± 0.03 | 0.59 ± 0.14 | 0.50 ± 0.06 | 0.45 ± 0.06 | 0.44 ± 0.02 | 6.26 ± 9.98 | 0.46 ± 0.02 |
| C 16:0 | 20.60 ± 0.95 | 17.98 ± 0.06 | 19.55 ± 0.34 | 19.90 ± 0.66 | 16.47 ± 0.70 | 15.73 ± 0.99 | 16.37 ± 1.49 | 16.55 ± 0.62 | 12.87 ± 10.97 | 18.29 ± 0.26 |
| C 16:1ω9 | 0.41 ± 0.11 | 0.31 ± 0.03 | 0.36 ± 0.02 | 0.33 ± 0.03 | 0.28 ± 0.07 | 0.19 ± 0.16 | 0.61 ± 0.28 | 0.31 ± 0.04 | 2.32 ± 3.50 | 0.27 ± 0.02 |
| C 16:1ω7 | 17.30 ± 0.50 | 16.26 ± 1.18 | 9.50 ± 0.75 | 7.96 ± 0.98 | 3.76 ± 0.62 | 4.57 ± 1.98 | 16.59 ± 1.99 | 11.43 ± 0.89 | 6.09 ± 0.23 | 6.15 ± 0.13 |
| C 17:0 | 0.49 ± 0.02 | 0.46 ± 0.05 | 0.53 ± 0.08 | 0.61 ± 0.13 | 1.13 ± 0.13 | 1.05 ± 0.12 | 0.78 ± 0.07 | 0.82 ± 0.08 | 0.69 ± 0.10 | 0.65 ± 0.05 |
| C DMA 18:0 | 1.97 ± 0.81 | 2.95 ± 1.14 | 3.21 ± 0.16 | 3.52 ± 0.07 | 7.78 ± 0.65 | 7.45 ± 1.75 | 3.33 ± 0.63 | 6.35 ± 0.28 | 3.83 ± 0.47 | 4.20 ± 0.43 |
| C 18:0 | 3.97 ± 0.15 | 3.72 ± 0.27 | 4.28 ± 0.18 | 4.28 ± 0.21 | 4.76 ± 0.56 | 4.65 ± 0.84 | 4.53 ± 0.32 | 4.69 ± 0.51 | 3.80 ± 0.50 | 3.46 ± 0.13 |
| C 18:1ω9 | 1.93 ± 0.09 | 1.79 ± 0.04 | 2.79 ± 0.06 | 2.68 ± 0.13 | 1.31 ± 0.15 | 1.75 ± 0.56 | 1.75 ± 0.20 | 1.40 ± 0.08 | 2.37 ± 0.10 | 2.20 ± 0.11 |
| C 18:1ω7 | 2.99 ± 0.08 | 2.68 ± 0.00 | 2.60 ± 0.12 | 2.74 ± 0.05 | 1.76 ± 0.11 | 1.93 ± 0.20 | 3.73 ± 0.35 | 3.33 ± 0.28 | 2.28 ± 0.02 | 2.21 ± 0.05 |
| C 18:2ω6 | 1.61 ± 0.05 | 1.72 ± 0.07 | 2.52 ± 0.08 | 2.67 ± 0.10 | 1.28 ± 0.09 | 1.44 ± 0.32 | 1.32 ± 0.10 | 1.00 ± 0.07 | 2.26 ± 0.12 | 2.29 ± 0.10 |
| C 18:3ω6 | 0.05 ± 0.10 | 0.21 ± 0.05 | 0.21 ± 0.01 | 0.21 ± 0.01 | 0.83 ± 0.10 | 0.79 ± 0.17 | 0.18 ± 0.15 | 0.59 ± 0.04 | 0.28 ± 0.05 | 0.24 ± 0.09 |
| C 18:3ω3 | 1.07 ± 0.01 | 1.12 ± 0.02 | 1.61 ± 0.06 | 1.40 ± 0.12 | 1.15 ± 0.23 | 1.18 ± 0.34 | 1.01 ± 0.06 | 0.81 ± 0.01 | 1.47 ± 0.11 | 1.28 ± 0.06 |
| C 18:4ω3 | 3.15 ± 0.09 | 3.60 ± 0.34 | 4.13 ± 0.20 | 3.45 ± 0.30 | 2.38 ± 0.63 | 2.39 ± 0.80 | 3.16 ± 0.18 | 2.78 ± 0.07 | 3.77 ± 0.22 | 3.30 ± 0.18 |
| C 20:1ω11 | 0.84 ± 0.01 | 0.81 ± 0.05 | 1.01 ± 0.22 | 1.21 ± 0.11 | 1.00 ± 0.14 | 1.21 ± 0.13 | 0.83 ± 0.12 | 0.92 ± 0.04 | 1.22 ± 0.04 | 1.08 ± 0.04 |
| C 20:1ω9 | 1.24 ± 0.07 | 1.12 ± 0.17 | 1.76 ± 0.11 | 1.86 ± 0.11 | 2.73 ± 0.16 | 3.05 ± 0.28 | 1.35 ± 0.07 | 1.57 ± 0.15 | 1.91 ± 0.14 | 1.82 ± 0.10 |
| C 20:1ω7 | 0.78 ± 0.06 | 0.68 ± 0.06 | 0.80 ± 0.11 | 0.80 ± 0.09 | 0.94 ± 0.03 | 1.02 ± 0.09 | 1.31 ± 0.09 | 1.56 ± 0.09 | 0.80 ± 0.04 | 0.74 ± 0.04 |
| C 20:2NMI1 | 0.98 ± 0.01 | 0.90 ± 0.20 | 1.58 ± 0.21 | 1.77 ± 0.15 | 1.92 ± 0.17 | 2.45 ± 0.12 | 1.15 ± 0.15 | 1.34 ± 0.07 | 1.93 ± 0.12 | 1.75 ± 0.14 |
| C 20:2NMI2 | 0.36 ± 0.01 | 0.36 ± 0.06 | 0.37 ± 0.05 | 0.47 ± 0.09 | 0.34 ± 0.01 | 0.50 ± 0.08 | 0.29 ± 0.07 | 0.33 ± 0.03 | 0.52 ± 0.03 | 0.49 ± 0.03 |
| C 20:2ω6 | 0.45 ± 0.02 | 0.45 ± 0.03 | 0.81 ± 0.05 | 0.87 ± 0.06 | 0.80 ± 0.06 | 0.92 ± 0.13 | 0.53 ± 0.02 | 0.54 ± 0.01 | 0.86 ± 0.07 | 0.82 ± 0.03 |
| C 20:4ω6 | 1.69 ± 0.00 | 1.75 ± 0.11 | 1.20 ± 0.05 | 1.35 ± 0.03 | 2.48 ± 0.24 | 2.64 ± 0.55 | 1.20 ± 0.21 | 1.45 ± 0.18 | 1.35 ± 0.21 | 1.56 ± 0.12 |
| C 20:4ω3 | 0.34 ± 0.06 | 0.33 ± 0.18 | 0.38 ± 0.09 | 0.31 ± 0.02 | 0.31 ± 0.05 | 0.28 ± 0.25 | 0.53 ± 0.01 | 0.57 ± 0.07 | 0.40 ± 0.01 | 0.34 ± 0.05 |
| C 20:5ω3 | 14.41 ± 0.90 | 16.56 ± 0.69 | 13.14 ± 0.53 | 11.39 ± 0.46 | 13.91 ± 0.98 | 12.51 ± 0.77 | 18.02 ± 2.57 | 19.00 ± 1.96 | 13.79 ± 0.74 | 15.61 ± 0.36 |
| C 22:0 | 0.24 ± 0.00 | 0.24 ± 0.01 | 0.23 ± 0.02 | 0.24 ± 0.02 | 0.08 ± 0.14 | 0.06 ± 0.10 | 0.22 ± 0.02 | 0.27 ± 0.02 | 0.20 ± 0.01 | 0.20 ± 0.03 |
| C 22:2NMID1 | 0.19 ± 0.01 | 0.18 ± 0.04 | 0.32 ± 0.05 | 0.43 ± 0.06 | 0.47 ± 0.02 | 0.66 ± 0.05 | 0.17 ± 0.02 | 0.21 ± 0.02 | 0.50 ± 0.04 | 0.43 ± 0.06 |
| C 22:2NMID2 | 1.90 ± 0.06 | 1.61 ± 0.25 | 0.99 ± 0.44 | 1.95 ± 0.24 | 2.74 ± 0.09 | 3.41 ± 0.23 | 1.80 ± 0.24 | 2.58 ± 0.17 | 1.70 ± 0.11 | 1.78 ± 0.20 |
| C 22:3 NMIT | 0.31 ± 0.00 | 0.30 ± 0.05 | 0.47 ± 0.10 | 0.73 ± 0.09 | 1.00 ± 0.11 | 1.26 ± 0.08 | 0.25 ± 0.02 | 0.32 ± 0.06 | 0.78 ± 0.05 | 0.78 ± 0.14 |
| C 22:4ω6 | 0.72 ± 0.02 | 0.89 ± 0.00 | 0.83 ± 0.02 | 0.83 ± 0.02 | 0.83 ± 0.05 | 0.84 ± 0.06 | 0.91 ± 0.10 | 0.99 ± 0.07 | 0.97 ± 0.05 | 1.05 ± 0.09 |
| C 22:5ω6 | 0.27 ± 0.00 | 0.28 ± 0.06 | 0.32 ± 0.02 | 0.38 ± 0.05 | 0.51 ± 0.01 | 0.52 ± 0.10 | 0.34 ± 0.05 | 0.41 ± 0.05 | 0.27 ± 0.04 | 0.26 ± 0.01 |
| C 22:5ω3 | 0.68 ± 0.03 | 0.77 ± 0.09 | 0.90 ± 0.11 | 0.95 ± 0.05 | 1.39 ± 0.18 | 0.97 ± 0.73 | 0.98 ± 0.07 | 1.40 ± 0.07 | 1.23 ± 0.13 | 1.18 ± 0.04 |
| C 22:6ω3 | 10.60 ± 0.66 | 12.12 ± 0.74 | 19.01 ± 1.15 | 20.55 ± 0.96 | 23.29 ± 1.97 | 22.48 ± 2.03 | 8.24 ± 1.55 | 10.51 ± 0.89 | 20.59 ± 1.01 | 21.91 ± 0.70 |

Table S5. Spatial and temporal variations of the fatty acid (FA) composition of the digestive gland (n = 30). The content is expressed as relative percentage of total FA (mean ± SD). The – indicates the FA was below the detection limit.

| FAME | Summer 2010 | | Autumn 2010 | | Winter 2011 | | Spring 2011 | | Autumn 2011 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 |
| SAFA | 33.61 ± 1.46 | 30.16 ± 0.47 | 29.18 ± 0.67 | 29.2 ± 0.76 | 24.82 ± 1.54 | 23.59 ± 0.67 | 30.41 ± 3.00 | 28.29 ± 1.66 | 26.53 ± 1.41 | 26.28 ± 0.11 |
| MUFA | 25.51 ± 0.76 | 23.66 ± 0.94 | 18.82 ± 0.94 | 17.57 ± 0.78 | 11.78 ± 0.86 | 13.71 ± 2.76 | 26.18 ± 2.81 | 20.52 ± 1.45 | 16.99 ± 3.70 | 14.46 ± 0.07 |
| PUFA | 38.89 ± 1.79 | 43.22 ± 0.28 | 48.79 ± 1.43 | 49.71 ± 1.04 | 55.63 ± 2.00 | 55.24 ± 2.05 | 40.07 ± 5.20 | 44.84 ± 3.03 | 52.65 ± 2.05 | 55.06 ± 0.50 |
| DMA | 1.97 ± 0.81 | 2.95 ± 1.14 | 3.21 ± 0.16 | 3.52 ± 0.07 | 7.78 ± 0.65 | 7.45 ± 1.75 | 3.33 ± 0.63 | 6.35 ± 0.28 | 3.83 ± 0.47 | 4.20 ± 0.43 |
| ω3 | 30.29 ± 1.70 | 34.53 ± 0.40 | 39.17 ± 1.50 | 38.06 ± 0.60 | 42.42 ± 2.35 | 39.81 ± 1.68 | 31.94 ± 4.39 | 35.07 ± 2.98 | 41.24 ± 1.55 | 43.62 ± 1.09 |
| ω6 | 4.82 ± 0.06 | 5.312 ± 0.19 | 5.89 ± 0.05 | 6.30 ± 0.08 | 6.73 ± 0.26 | 7.16 ± 0.38 | 4.47 ± 0.40 | 4.98 ± 0.21 | 5.98 ± 0.40 | 6.22 ± 0.10 |
| ω7 | 21.08 ± 0.66 | 19.62 ± 1.10 | 12.91 ± 0.94 | 11.50 ± 0.88 | 6.46 ± 0.70 | 7.52 ± 2.20 | 21.63 ± 2.41 | 16.32 ± 1.22 | 9.17 ± 0.22 | 9.09 ± 0.11 |
| ω9 | 3.59 ± 0.10 | 3.22 ± 0.10 | 4.91 ± 0.18 | 4.86 ± 0.01 | 4.32 ± 0.24 | 4.99 ± 0.46 | 3.72 ± 0.54 | 3.28 ± 0.26 | 6.6 ± 3.45 | 4.29 ± 0.09 |
| ω11 | 0.84 ± 0.01 | 0.81 ± 0.05 | 1.01 ± 0.22 | 1.21 ± 0.11 | 1.00 ± 0.14 | 1.21 ± 0.13 | 0.83 ± 0.12 | 0.92 ± 0.04 | 1.22 ± 0.04 | 1.08 ± 0.04 |
| PUFA ω3 | 30.29 ± 1.70 | 34.53 ± 0.4 | 39.17 ± 1.50 | 38.06 ± 0.60 | 42.42 ± 2.35 | 39.81 ± 1.68 | 31.94 ± 4.39 | 35.07 ± 2.98 | 41.24 ± 1.55 | 43.62 ± 1.09 |
| ω3/ω6 | 6.27 ± 0.31 | 6.50 ± 0.31 | 6.65 ± 0.24 | 6.04 ± 0.16 | 6.31 ± 0.53 | 5.57 ± 0.13 | 7.13 ± 0.44 | 7.03 ± 0.30 | 6.91 ± 0.30 | 7.02 ± 0.27 |
| NMI | 3.77 ± 0.08 | 3.37 ± 0.49 | 3.73 ± 0.09 | 5.35 ± 0.60 | 6.48 ± 0.26 | 8.28 ± 0.29 | 3.67 ± 0.47 | 4.79 ± 0.25 | 5.43 ± 0.23 | 5.22 ± 0.52 |
| NMID | 3.45 ± 0.08 | 3.06 ± 0.44 | 3.26 ± 0.17 | 4.62 ± 0.51 | 5.48 ± 0.15 | 7.02 ± 0.23 | 3.42 ± 0.46 | 4.47 ± 0.23 | 4.65 ± 0.19 | 4.44 ± 0.38 |
| NMIT | 0.32 ± 0.00 | 0.30 ± 0.05 | 0.47 ± 0.10 | 0.73 ± 0.09 | 1.00 ± 0.11 | 1.26 ± 0.08 | 0.25 ± 0.02 | 0.32 ± 0.06 | 0.78 ± 0.05 | 0.78 ± 0.14 |

Table S6. Spatial and temporal variations of the fatty acid (FA) composition of the digestive gland (n = 30). The content is expressed as relative percentage of total FA (mean ± SD). The – indicates the FA was below the detection limit. SAFA = saturated FA, MUFA = monounsaturated FA, PUFA = polyunsaturated FA, DMA= dimethyl acetals FA, PUFA ratio for the ω3 and ω6 series, NMI= non-methylene-interrupted FA, NMID= NMI dienoic FA, NMIT= NMI trienoic FA.

| FAME | Summer 2010 | | Autumn 2010 | | Winter 2011 | | Spring 2011 | | Autumn 2011 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 |
| C 14:0 | 7.52 ± 0.08 | 6.74 ± 0.62 | 5.03 ± 0.82 | 4.57 ± 1.23 | 6.63 ± 0.75 | 6.04 ± 3.19 | 5.84 ± 1.42 | 7.18 ± 0.70 | 4.06 ± 2.75 | 5.24 ± 0.19 |
| C 15:0 | 1.52 ± 0.07 | 1.67 ± 0.08 | 1.32 ± 0.05 | 1.44 ± 0.48 | 2.36 ± 0.14 | 2.91 ± 0.20 | 0.61 ± 0.07 | 0.84 ± 0.05 | 1.28 ± 0.19 | 1.07 ± 0.06 |
| C 16:0 | 32.83 ± 0.11 | 33.6 ± 1.71 | 30.88 ± 6.79 | 26.89 ± 3.28 | 28.64 ± 0.67 | 26.25 ± 1.19 | 23.1 ± 0.66 | 23.81 ± 0.46 | 29.86 ± 0.94 | 28.02 ± 0.11 |
| C 16:1ω9 | 1.92 ± 0.08 | 2.57 ± 0.43 | 1.90 ± 0.53 | 1.92 ± 0.69 | 4.38 ± 0.47 | 7.16 ± 0.25 | 0.91 ± 0.17 | 1.63 ± 0.28 | 2.94 ± 0.36 | 2.14 ± 0.25 |
| C 16:1ω7 | 13.84 ± 2.33 | 12.18 ± 0.81 | 6.95 ± 1.29 | 5.96 ± 2.39 | 8.83 ± 1.19 | 11.87 ± 0.85 | 5.81 ± 1.67 | 5.30 ± 0.52 | 6.59 ± 0.79 | 6.10 ± 0.15 |
| C 17:0 | 1.93 ± 0.06 | 2.11 ± 0.01 | 2.15 ± 0.18 | 3.38 ± 0.93 | 1.89 ± 0.27 | 1.94 ± 0.38 | 0.68 ± 0.04 | 0.92 ± 0.06 | 1.43 ± 0.12 | 1.03 ± 0.06 |
| C DMA 18:0 | 3.00 ± 0.10 | 1.96 ± 0.19 | 2.81 ± 0.31 | 1.13 ± 0.54 | 1.85 ± 0.43 | 2.38 ± 0.21 | 1.34 ± 0.88 | 1.14 ± 0.02 | 2.21 ± 0.40 | 1.43 ± 0.11 |
| C 18:0 | 3.92 ± 0.11 | 7.24 ± 1.65 | 11.22 ± 2.85 | 25.5 ± 13.33 | 13.69 ± 2.92 | 7.10 ± 1.82 | 1.60 ± 0.23 | 2.53 ± 0.58 | 4.98 ± 1.19 | 6.19 ± 0.50 |
| C 18:1ω9 | 3.51 ± 0.10 | 4.56 ± 1.51 | 5.98 ± 0.63 | 5.02 ± 0.43 | 6.90 ± 1.68 | 5.42 ± 2.46 | 3.31 ± 0.54 | 4.85 ± 0.96 | 7.38 ± 0.10 | 5.34 ± 0.38 |
| C 18:1ω7 | 4.16 ± 0.05 | 3.92 ± 0.27 | 2.17 ± 1.77 | 3.54 ± 1.36 | 2.30 ± 0.28 | 4.39 ± 0.35 | 1.90 ± 0.55 | 1.98 ± 0.12 | 2.23 ± 0.25 | 2.51 ± 0.23 |
| C 18:2ω6 | 1.49 ± 0.06 | 1.53 ± 0.20 | 1.46 ± 0.17 | 1.69 ± 0.68 | 2.11 ± 0.40 | 2.28 ± 0.26 | 0.97 ± 0.10 | 0.96 ± 0.06 | 2.10 ± 0.10 | 1.61 ± 0.05 |
| C 18:3ω6 | 0.21 ± 0.01 | 0.10 ± 0.08 | 0.26 ± 0.03 | - | - | - | 0.11 ± 0.10 | - | 0.18 ± 0.05 | 0.12 ± 0.02 |
| C 18:3ω3 | 0.55 ± 0.03 | 0.58 ± 0.07 | 0.71 ± 0.16 | 0.54 ± 0.23 | 0.93 ± 0.17 | 0.60 ± 0.53 | 0.62 ± 0.08 | 0.63 ± 0.01 | 0.86 ± 0.05 | 0.46 ± 0.01 |
| C 18:4ω3 | 1.10 ± 0.06 | 1.21 ± 0.12 | 1.93 ± 0.52 | 1.16 ± 0.65 | 1.92 ± 0.16 | 1.85 ± 0.10 | 6.59 ± 0.82 | 6.00 ± 0.17 | 3.20 ± 0.18 | 3.91 ± 0.19 |
| C 20:1ω11 | 1.35 ± 0.09 | 0.84 ± 0.04 | 1.10 ± 0.30 | 0.48 ± 0.24 | 0.92 ± 0.33 | 0.92 ± 0.10 | 0.70 ± 0.28 | 0.52 ± 0.02 | 0.78 ± 0.12 | 0.49 ± 0.06 |
| C 20:1ω9 | 1.47 ± 0.10 | 1.15 ± 0.12 | 1.28 ± 0.55 | 0.45 ± 0.19 | 0.83 ± 0.43 | 0.81 ± 0.02 | 0.48 ± 0.32 | 0.29 ± 0.01 | 0.80 ± 0.14 | 0.81 ± 0.07 |
| C 20:1ω7 | 1.17 ± 0.01 | 0.89 ± 0.27 | 0.61 ± 0.29 | - | 0.21 ± 0.37 | - | 0.47 ± 0.53 | 0.25 ± 0.03 | 0.32 ± 0.02 | 0.24 ± 0.04 |
| C 20:2NMI1 | 0.95 ± 0.03 | 0.69 ± 0.03 | 1.43 ± 0.27 | 0.48 ± 0.26 | 0.96 ± 0.44 | 0.87 ± 0.03 | 0.43 ± 0.33 | 0.27 ± 0.02 | 0.99 ± 0.06 | 0.58 ± 0.04 |
| C 20:2NMI2 | 0.35 ± 0.03 | 0.24 ± 0.03 | 0.30 ± 0.07 | - | - | - | 0.11 ± 0.19 | - | 0.04 ± 0.08 | 0.13 ± 0.01 |
| C 20:2ω6 | 0.37 ± 0.04 | 0.37 ± 0.05 | 0.45 ± 0.08 | - | - | - | 0.19 ± 0.10 | 0.17 ± 0.02 | 0.27 ± 0.04 | 0.15 ± 0.02 |
| C 20:4ω6 | 1.15 ± 0.09 | 2.10 ± 0.13 | 2.00 ± 0.49 | 6.66 ± 4.14 | 3.76 ± 0.61 | 4.66 ± 0.52 | 0.71 ± 0.12 | 0.80 ± 0.11 | 1.35 ± 0.98 | 2.83 ± 0.16 |
| C 20:4ω3 | 0.26 ± 0.03 | 0.23 ± 0.11 | 0.14 ± 0.12 | - | - | - | 0.13 ± 0.11 | 0.15 ± 0.02 | 0.05 ± 0.09 | 0.03 ± 0.05 |
| C 20:5ω3 | 5.44 ± 0.09 | 6.00 ± 1.10 | 3.98 ± 1.32 | 2.88 ± 1.33 | 3.53 ± 0.31 | 5.43 ± 0.77 | 4.61 ± 2.13 | 4.73 ± 0.29 | 3.67 ± 0.53 | 2.94 ± 0.15 |
| C 22:0 | 0.37 ± 0.04 | 0.32 ± 0.07 | 2.29 ± 0.77 | 1.14 ± 0.61 | 2.10 ± 0.32 | 1.08 ± 0.10 | 16.27 ± 3.44 | 13.77 ± 0.31 | 7.57 ± 0.57 | 11.09 ± 0.56 |
| C 22:2NMID1 | 0.22 ± 0.01 | 0.12 ± 0.11 | 0.44 ± 0.15 | - | - | - | 0.06 ± 0.10 | 0.04 ± 0.08 | 0.38 ± 0.05 | 0.16 ± 0.01 |
| C 22:2NMID2 | 2.30 ± 0.06 | 1.35 ± 0.17 | 2.28 ± 0.24 | 0.81 ± 0.51 | 1.48 ± 0.59 | 1.21 ± 0.14 | 0.82 ± 0.62 | 0.61 ± 0.03 | 1.48 ± 0.24 | 0.75 ± 0.05 |
| C 22:3 NMIT | 0.25 ± 0.03 | 0.31 ± 0.06 | 0.53 ± 0.07 | - | - | - | 0.06 ± 0.11 | 0.06 ± 0.10 | 0.39 ± 0.07 | 0.18 ± 0.02 |
| C 22:4ω6 | 0.27 ± 0.05 | 0.28 ± 0.03 | 0.25 ± 0.09 | - | - | - | 0.11 ± 0.19 | 0.22 ± 0.02 | - | - |
| C 22:5ω6 | 0.20 ± 0.08 | 0.04 ± 0.07 | 0.14 ± 0.14 | - | - | - | 0.06 ± 0.11 | 0.05 ± 0.09 | 0.30 ± 0.03 | 0.16 ± 0.11 |
| C 22:5ω3 | 0.60 ± 0.10 | 0.60 ± 0.07 | 0.57 ± 0.31 | 0.30 ± 0.39 | 0.80 ± 0.03 | 2.62 ± 2.56 | 0.48 ± 0.17 | 0.47 ± 0.03 | 0.75 ± 0.19 | 0.52 ± 0.10 |
| C 22:6ω3 | 3.10 ± 0.10 | 4.48 ± 0.64 | 7.47 ± 2.67 | 4.07 ± 2.16 | 2.98 ± 0.22 | 2.19 ± 0.05 | 20.91 ± 2.70 | 19.84 ± 0.98 | 11.54 ± 0.64 | 13.76 ± 0.57 |

Table S7. Spatial and temporal variations of the fatty acid (FA) composition of the feces (n = 30). The content is expressed as relative percentage of total FA (mean ± SD). The – indicates the FA was below the detection limit.

| FAME | Summer 2010 | | Autumn 2010 | | Winter 2011 | | Spring 2011 | | Autumn 2011 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 | P-46 | P-14 |
| SAFA | 48.25 ± 0.25 | 51.69 ± 0.55 | 52.88 ± 6.30 | 62.91 ± 13.3 | 55.31 ± 3.51 | 45.31 ± 3.03 | 48.10 ± 4.81 | 49.05 ± 1.51 | 49.17 ± 2.74 | 52.63 ± 0.57 |
| MUFA | 30.29 ± 0.21 | 26.12 ± 1.48 | 19.99 ± 1.53 | 17.38 ± 4.46 | 24.38 ± 3.02 | 30.57 ± 1.32 | 13.58 ± 2.93 | 14.82 ± 1.25 | 21.05 ± 0.53 | 17.64 ± 0.59 |
| PUFA | 19.20 ± 0.30 | 20.23 ± 1.89 | 24.33 ± 5.16 | 18.58 ± 8.77 | 18.45 ± 0.63 | 21.73 ± 2.61 | 36.98 ± 2.01 | 35.00 ± 1.20 | 27.57 ± 2.08 | 28.29 ± 0.37 |
| DMA | 3.26 ± 0.24 | 1.96 ± 0.19 | 2.81 ± 0.31 | 1.13 ± 0.54 | 1.85 ± 0.43 | 2.38 ± 0.21 | 1.34 ± 0.88 | 1.14 ± 0.02 | 2.21 ± 0.40 | 1.43 ± 0.11 |
| ω3 | 11.54 ± 0.46 | 13.10 ± 1.91 | 14.78 ± 4.95 | 8.95 ± 4.58 | 10.15 ± 0.74 | 12.71 ± 2.09 | 33.34 ± 1.77 | 31.83 ± 1.28 | 20.07 ± 1.23 | 21.63 ± 0.65 |
| ω6 | 3.86 ± 0.14 | 4.41 ± 0.11 | 4.57 ± 0.83 | 8.35 ± 3.68 | 5.87 ± 0.74 | 6.95 ± 0.77 | 2.15 ± 0.63 | 2.19 ± 0.12 | 4.20 ± 1.09 | 4.86 ± 0.26 |
| ω7 | 21.94 ± 0.06 | 16.99 ± 0.60 | 9.72 ± 0.41 | 9.50 ± 3.75 | 11.35 ± 1.73 | 16.26 ± 1.01 | 8.18 ± 2.74 | 7.53 ± 0.67 | 9.14 ± 1.01 | 8.86 ± 0.30 |
| ω9 | 6.93 ± 0.07 | 8.29 ± 1.03 | 9.16 ± 1.03 | 7.39 ± 0.48 | 12.11 ± 1.59 | 13.39 ± 2.35 | 4.70 ± 0.53 | 6.76 ± 1.22 | 11.13 ± 0.51 | 8.29 ± 0.66 |
| ω11 | 1.68 ± 0.32 | 0.84 ± 0.04 | 1.10 ± 0.30 | 0.48 ± 0.24 | 0.92 ± 0.33 | 0.92 ± 0.10 | 0.70 ± 0.28 | 0.52 ± 0.02 | 0.78 ± 0.12 | 0.49 ± 0.06 |
| PUFA ω3 | 11.54 ± 0.46 | 13.10 ± 1.91 | 14.78 ± 4.95 | 8.95 ± 4.58 | 10.15 ± 0.74 | 12.71 ± 2.09 | 33.34 ± 1.77 | 31.83 ± 1.28 | 20.07 ± 1.23 | 21.63 ± 0.65 |
| ω3/ω6 | 2.99 ± 0.01 | 2.96 ± 0.37 | 3.19 ± 0.71 | 1.05 ± 0.35 | 1.74 ± 0.11 | 1.83 ± 0.19 | 16.33 ± 4.41 | 14.55 ± 1.07 | 5.03 ± 1.50 | 4.46 ± 0.37 |
| NMI | 4.30 ± 0.20 | 2.72 ± 0.15 | 4.97 ± 0.66 | 1.28 ± 0.77 | 2.44 ± 1.02 | 2.08 ± 0.14 | 1.48 ± 1.35 | 0.98 ± 0.12 | 3.30 ± 0.34 | 1.80 ± 0.04 |
| NMID | 3.92 ± 0.08 | 2.41 ± 0.15 | 4.44 ± 0.72 | 1.28 ± 0.77 | 2.44 ± 1.02 | 2.08 ± 0.14 | 1.42 ± 1.24 | 0.92 ± 0.12 | 2.90 ± 0.28 | 1.62 ± 0.06 |
| NMIT | 0.38 ± 0.12 | 0.31 ± 0.06 | 0.53 ± 0.07 | - | - | - | 0.06 ± 0.11 | 0.06 ± 0.10 | 0.39 ± 0.07 | 0.18 ± 0.02 |

Table S8. Spatial and temporal variations of the fatty acid (FA) composition of the feces (n = 30). The content is expressed as relative percentage of total FA (mean ± SD). The − indicates the FA was below the detection limit. SAFA = saturated FA, MUFA = monounsaturated FA, PUFA = polyunsaturated FA, DMA= dimethyl acetals FA, PUFA ratio for the ω3 and ω6 series, NMI= non-methylene-interrupted FA, NMID= NMI dienoic FA, NMIT= NMI trienoic FA.

| FAME | Fish feed |
|---|---|
| C 14:0 | 5.71 ± 0.07 |
| C 15:0 | 0.54 ± 0.02 |
| C 16:0 | 15.94 ± 0.15 |
| C 17:0 | 0.74 ± 0.00 |
| C 18:0 | 4.46 ± 0.07 |
| C 22:0 | 0.02 ± 0.03 |
| C DMA 18:0 | 0.78 ± 0.01 |
| Σ SAFA | 27.40 ± 0.22 |
| C 16:1ω9 | 0.23 ± 0.02 |
| C 16:1ω7 | 7.60 ± 0.06 |
| C 18:1ω9 | 18.14 ± 0.15 |
| C 18:1ω7 | 3.25 ± 0.05 |
| C 20:1ω11 | 0.15 ± 0.01 |
| C 20:1ω9 | 1.18 ± 0.02 |
| C 20:1ω7 | 0.23 ± 0.00 |
| Σ MUFA | 30.78 ± 0.24 |
| C 18:2n6 | 9.00 ± 0.09 |
| C 18:3n3 | 2.30 ± 0.03 |
| C 18:4n3 | 1.61 ± 0.02 |
| C 20:2NMI1 | 0.20 ± 0.01 |
| C 20:2ω6 | 0.24 ± 0.02 |
| C 20:4ω6 | 1.26 ± 0.71 |
| C 20:4ω3 | 0.70 ± 0.01 |
| C 20:5ω3 | 11.60 ± 0.14 |
| C 22:4ω6 | 0.89 ± 0.00 |
| C 22:5ω6 | 0.72 ± 0.02 |
| C 22:5ω3 | 1.63 ± 0.01 |
| C 22:6ω3 | 10.91 ± 0.18 |
| Σ PUFA | 41.05 ± 0.45 |
| ω3/ω6 | 2.38 ± 0.18 |

Table S9. Seasonal content of the principal fatty acid (FA) groups and classes observed for the fish feed samples (n = 3). The content is expressed as relative percentage of total FA (mean ± SD).

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