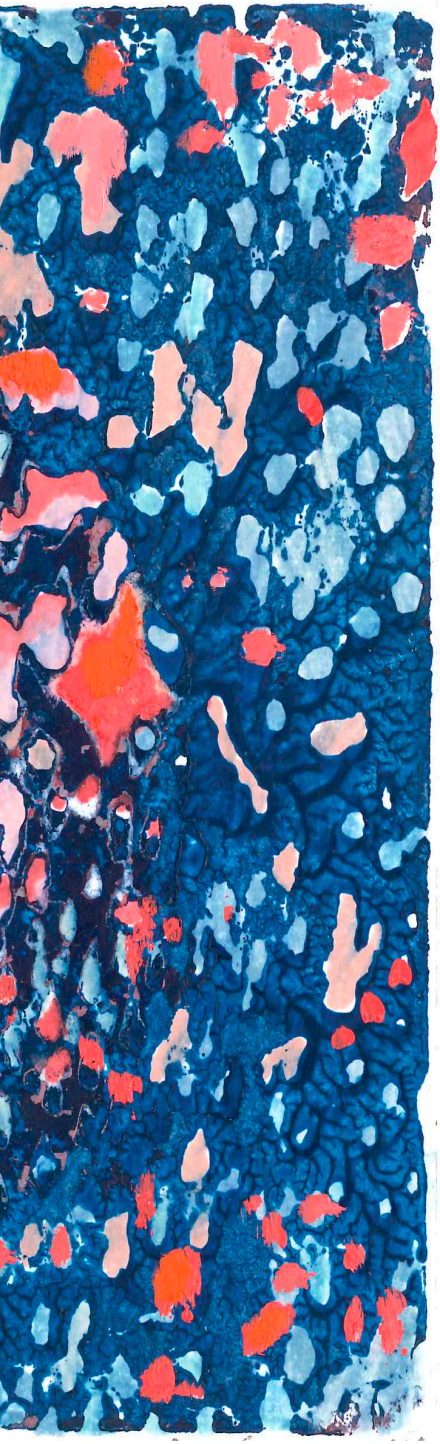

FEASIBILITY OF MUSSEL (*Mytilus galloprovincialis* Lmk.) AQUACULTURE IN THE OPEN OCEAN WATERS OF THE SE BAY OF BISCAY (BASQUE COUNTRY).

Katerin Azpeitia Abarrategi

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Bizkaiko Golkoan erauzketa-arrantzaren ustiapen mailaren igoerak eta harrapakinen bolumenaren jaitsierak, Euskal Herriko erkidegoaren arrantza jarduerak dibertsifikatuko dituen eta lehen sektorean lana sortuko duen alternatibak bilatzera behartzen gaituzte. Etorkizunean aurreikusten den giza populazioaren hazkundeak elikagaien ekoizpena handitzea eskatzen du eta bereziki, animali jatorrizko proteinen ekoizpena. Mytilus galloprovincialis Lmk. aukera naturala da Bizkaiko Golko hego-ekialdean eta lan honek Euskal herrian muskuilu industria bat aurrera ateratzeko baldintza biologikoak betetzen diren aztertzea du helburu.

Muskuiluen kultiboa ekosistemak jasan dezakeen euste-ahalmenak baldintzatzen du, eta kultiboaren arrakasta, besteak beste, muskuilu-espezieak, landatutako animaliaren bizi zikloak eta kultiboa gauzatzen den ingurugiroaren faktore sozio-ekonomiko espezifikoek baldintzatuko dute. Kostalde eremuetan akuikultura gauzatzeko lur erabilgarria gero eta mugatuagoa da, jurisdikzio eskumena zorrozki betearazten da bertan eta alokairu mugatua izaten du. Gainera, eskualde askoren itsasertzaren geografia, bateak eta longline sistemak aterpetzeko ez aski izan daiteke. Bestalde, seston hornikuntza mugatu batek edo/eta ur kutsatuek kostaldeko muskuiluen akuikultura gauzatzeko faktore mugatzaileak izan daitezke.

Itsaso zabalean ur azpiko bibalboen akuikulturan, biologia zein ingeniaritza arrakastatsuen adibide urri daude (Langan, 2001; Langan and Horton, 2003; Buck, 2007). Longline sistema, Japonen ostrak kultibo esekietatik garatutako metodo bat da, ur gaineko edota ur azpiko longlines egina, ainguren bitartez tokira finkatua, eta boia zein flotagailuen bitartez sostengatzen dena. Baldintza energetiko handiko itsasoetan, Bizkaiko Golkoaren hego-ekialdeko itsasoak bezalakoetan, urpeko longline sistema aukera bakarra kontsideratzen da.

Horregatik, ikerketa honek esekitako muskuiluak kultibatzeko itsasertzeko lur eremuetan era tradizionalaren alternatiba interesgarri bat ikertzen du. Ikerketa honek bi helburu nagusi ditu. Lehenengo helburua euskal kostaldean bizi diren muskuilu populazioaren ugalketa patroia eta larben kolonizazioaren patroia ebaluatzea da, kostaldeko muskuiluen kolonizazioa, itsaso zabaleko kultibo sistema hazi naturalekin hornitzeko ainakoa den jakiteko. Bigarren helburua, Bizkaiko Golkoaren hego ekialdean kultibo sistema pilotu batean, bi kultibo sakonerarekin, kultibatutako muskuiluen (Mytilus galloprovincialis Lmk.) gizentze tasa, biziraupen tasa eta produktuaren kalitatea ebaluatzea da, kultiboaren baldintzak merkatuaren eskariak akuikultura produkzio jasangarri eta oparo bat betetzeko gai diren jakiteko. Ikerketa honek bestalde bigarren mailako helburu bat du kultiboaren kudeaketa hobetzeko

asmoarekin, ikerketa honetako laginketa-estazioetan, itsasertzean zein itsaso zabalean, errendimendu biologiko horiek modulatzen dituzten ingurumen faktoreen harreman kausala ebaluatzea du helburu, hain zuzen ere.

Zazpi ataletan banatzen da lan hau. I Kapituluan, itsaso zabaleko akuikulturari buruzko sarrera orokor bat (gertaera historikoak, jatorria, abantailak eta desabantailak), eta muskuiluen biologiaren alderdi orokorrak aurkezten dira. Atal honetan, baita ere, lanaren helburuak eta probatu nahi den hipotesia aurkezten dira. II atalean euskal kostaldean 5 laginketa-estazioetan, hiru estuario eta bi itsasertzetan, bizi diren muskuilu populazioen ugalketa zikloa deskribatzen da. III atalean, euskal kostaldeko muskuiluen kolonizazio eta erreklutamendu patroia aztertzen da. IV eta V ataletan, Bizkaiko Golkoaren hego-ekialdean urpeko longline sistema pilotu batean, bi sakoneretara, gizendutako muskuiluen Mytilus galloprovincialis Lmk. sasoiko hazkuntza eta errendimendu biologikoak deskribatzen dira. IV atalean, kultibo sisteman urtaroen arabera bildutako muskuilu guztien produkzioa, eta muskuilu horien kalitatea, deskribatzen da. V atalean, kultibo sistema pilotuan guk landatukotako muskuilu hazi sortaren (sistemako sokak kolonizatu dituzten muskuilu hazi berriak estimaziotik ezabatuaz) benetako hazkuntzaren errendimendua deskribatzen da. Atal honek produkzioa eta biomasaren aldakuntzak

deskribatzen ditu ere. Gainera, kapitulu honetan urtaroka bildutako tamaina komertzial ezberdinetako muskuiluen kalitatea (muskuilu komertzial txikiak eta handiak) deskribatzen da. VI atalean, itsaso zabaleko sisteman bildutako tamaina handiko muskuilu komertzialen (>70mm) eta merkatuan eskuragarri dauden tamaina bereko muskuluen arteko kontsumitzaileen onarpenaren alderaketa deskribatzen da. VII atalean ikerketaren ondorioak laburbiltzen dira, etorkizunean egin daitezkeen ikerketa gehiago iradokitzen dira eta ikerketa honetan planteatzen den hipotesiari erantzuna ematen zaio.

Eusko jaurlaritzak ez dauka eskumenik Bizkaiko Golkoaren hego-ekialdeko itsaso zabalean, euskal kostaldearen iparraldean, elikagaien ekoizpenik burutzeko. Arrazoi hauengatik, Espainiar estatuari lizentziak eskatu zitzaizkion, urpeko akuikultura sistema bat instalatzeko kostaldetik 2 mila nautikora. Lizentziaren tramitazioak urte eta erdi baino gehiago iraun zuen. Arrazoi hauengatik, itsaso zabalean kultibo sistemaren ezarpena eta lehenagotik iragarritako kultiboaren denborak atzeratu egin dira. Hala ere, ikerketaren lehen zatia, kostaldeko laginketa-estazioetan, beste urtebetez luzatu da atzerapen hau gainditzeko. Ondoren, itsaso zabaleko laginketa-estazioetan, ikerketaren bigarren zatia zuzenki burutu da.

M. galloprovincialis Lmk-ren ugalketaren osasun egoera Euskal

Herriko kostaldeko 5 laginketa-estazioetan, 3 estuarioetan eta 2 itsasertzetan, 2 urte jarraian (2010 eta 2011) aztertu da. Sexu-ratioa, gonaden garapen faseak eta erreserba ehunen zikloa aztertu direlarik. Itsasertzeko bi laginketa-estazioak eta estuario inguruko 3 laginketa-estazioak gazitasun eta janari iturri alternatiboen mailaren arabera (e.g karbono organikoa) bereizi ziren. Euskal kostaldean urtaroak itsaso azaleraren tenperaturaren zikloa eta suspentsio solidoen zikloaren bitartez argi eta garbi bereizten dira. Azkenik, estuarioetako laginketa-estazioetan udaberrian eta udaran aurkitu diren a-klorofilaren pikoak. Itsasertzeko laginketa-estazioetan udazken eta neguan aurkitu dira piko txikienak.

Euskal kostaldean barrena gametogenesiaren hasiera negutik udazkenera ikusi da. Errutea, berriz, udaberritik uda-arte ikusi da. Errute garai ezberdinak ikusi dira laginketa-estazioaren arabera, eta laginketa-estazio batzuetan, baita ere ikusi da urtez urteko aldakortasuna. Sexuen arteko alderaketa aztertzean, bakarrik Mutriku itsasertz laginketa-estazioan ar gehiagoren aldeko sexu-ratioaren desbideratze garrantzitsu bat ikusi da eta Oiartzun estuario laginketa-estazioan, ikerketa osoan aurkitu diren bi indibiduo hermafrodita bakarrak bertan aurkitu dira. Errute ostean, uda amaieratik udazkenera, oozitoen atresia maila handitu egiten da eta ondorioz erreserba ehunen (ADG zelulak) maila

handitzen dela ikusten da. Beste erreserba ehunen (VCT zelulak) maila udazkenetik negura handitzen da. Hortaz esandako guztia kontutan harturik, laginketa-estazio hauetan bizi diren muskuiluek ugalketa-ziklo normal bat daukatela esan dezakegu. Bestalde, muskuilu hazien iturri bihurtu ahalko dira espero den muskuilu kultibo komertzialarentzat. Horrela eginez gero, eta ugaltze tasa maximo bat ziurtatu eta babestu nahiko balitz, muskuiluen bilketa euskal kostaldean udaberritik udara amaiera-arte saihestu beharko litzateke.

Mytilus galloprovincialis Lmk.-en kolonizazio eta erreklutamendu patroia euskal kostaldean barrena aztertu da ugalketaren osasun analisiarekin batera. Aurretik deskribatutako muskuilu populazioetatik gertu eta kaptaziorako sokak erabiliz aztertu zen kostaldeko erreklutamendua. Bi ikerketak elkarrekin burutzeak, muskuilu populazio basatien errute garaia eta lehen kolonizazioaren arteko erlazioa aurkitzea gaitu du. Lehen kolonizatzaileen ugaritasun handiena udara hasieratik udazkenera ikusi da. Hala ere, euskal kostaldean muskuilu gazteen erreklutamendua kolonizazio osteko prozesuek zehazten dute hasierako kolonizazio mailak baino, eta ondorioz, muskuilu basatien populazioak ez dira oso zabalak. Ekialdeko estuario laginketa-estazioek mendebaldeko estuario laginketa-estazioek baino kolonizazio maila altuagoa eta erreklutamendu tasa gorenagoa erakutsi dute. Itsasertzeko

laginketa-estazioek hasierako kolonizazio eta bukaerako erreklutamendu abundantzia-tasa tartekoak erakutsi dituztelarik. Ikertutako bi urteetan aurkitutako ezberdintasunak tenperatura, a-klorofilaren maila eta uraren mugimendu mailari egotziak izan dira. Erabiltzaileen arteko gatazka dela eta, euskal kostaldean muskuilu haziak biltzeko lur eremu erabilgarria mugatua izan daiteke. Hala ere, ikertutako laginketa-estazioetan muskuilu haziak bilduko balira, bereziki Bidasoa estuario laginketa-estazioan, itsaso zabaleko gizentze estazioetan landatu ahalko lirateke. Hortaz, hazi bilketa honek etorkizuneko hazi eskaria asetzen lagundu dezake. Laginketa-estazio hauek erabiliko balira, soken bilketa noiz egin zaindu beharko litzateke. Kolonizazio osteko prozesuak hobe kontrolatzeko asmoz, sokak udara amaieran edota udazkenean akuikultura instalazioetara eraman ahalko lirateke.

Ikerketa honen bigarren zatia itsaso zabaleko akuikultura ikertzea da. Honetarako, Mytilus galloprovincialis Lmk. muskuilu haziak populazio naturaletatik bildu ziren eta esekitako 24 gizentze soketara transferitu (eskuz egindako aletzearen bitartez) ziren. Gizentze sokak longline sistemako bi sakonera mailetatik (5m-ko eta 15m-ko sakoneretatik hain zuzen ere) 12:12 moduan banaturik jarri ziren. Muskuiluen hasierako tamaina eta dentsitatea 17.40 ± 5.07mm eta 681 ± 37 indibiduo/metro izan da. Muskuilu haziak 2013ko ekainetik 2014ko abuztu-arte

kultibatu dira. Laginketa bakoitzean, 4-5 hilero (eguraldiaren arabera), sakonera bakoitzetik ausazko 3 soka lagindu dira. Muskuiluen luzera eta abundantzia tasa, soken dentsitatea (indibidu/ metro) eta hilkortasun tasa estimatzeko erabili direlarik. Bestalde, lagindutako sakonera bakoitzean eta laginketa bakoitzeko, 36m sokan (3 soka) lagindutako tamainen maiztasun absolutua estimatu da.

Itsaso zabaleko kultibo sisteman Mytilus galloprovincialis Lmk.-en hazkuntza errendimendua oskolaren alometria, baldintza indizea eta muskuiluaren biokimika neurtuaz aztertu dira. Hazkuntza masan, luzera eta masaren arteko erlazio alometrikoaren bitartez neurtu zen, soken berehalako hazkuntza tasa (G) eta eguneko hazkuntza tasa (%G) ere neurtu delarik. Era berean, hazkuntza, luzeran, egunen eta luzeraren arteko erlazio esponentzialaren bitartez neurtu da. 5m-ko kultibo sakoneran muskuilu larben kolonizazioa aurkitu da. Guk landatutako muskuilu hazien benetako hazkuntza tasa kalkulatzeko, muskuiluak oskolaren luzeraren ara-behera 10mm luzera-klasetan sailkatu dira (klase tamaina konstanteak). Ebatzitako luzera-maiztasun taulak Gausiian osagaietan ebatzi ditugu, FISAT II programan dagoen Bhattacharya (1969) metodoaren bitartez. Ustezko adin-klase nagusiaren (guk landatutako hazi originalak) urtaroka ebatzitako luzera balio modalak, laginketa eta lagindutako sakonera bakoitzean, Von Bertanlanffy urtaro

hazkuntza funtzioa (VBSGF) ebazteko erabili dira. Muskuiluen kalitate karakteristikak (i.e., baldintza, biokimika eta gantz azidoen konposizioa) urtaroka aztertu dira sakonera bakoitzean bildutako muskuiluekin. Gainera, tamaina komertzial ezberdinetako (muskuilu komertzial txiki eta handiak; 50-70mm eta >70 mm) muskuiluen kalitate ezaugarriak urtaroka aztertu ditugu baita ere. Azkenik, itsaso zabalean kultibatutako "tamaina handiko muskuilu komertzialak" eta tamaina bereko eta gaur egun merkatuan topatu daitezkeen muskuilu komertzialekin alderatu dira, kontutan hartuta lehen aipaturiko baldintza indizea, biokimika eta gantz azidoen konposaketa analisiaz gain, kontsumitzaileen zentzumen ebaluazio baten bidez.

Itsasoaren tenperatura eta korronteak itsaso zabaleko laginketa-estazioan NEMO (Nucleus European Modelling of the Ocean) modeloaren bitartez lortu dira. Olatuei buruzko informazioa (olatuen altuera signifikatiboa, Hs, eta pikoaren aldia , Tp), bi Metocean boien bitartez lortu ziren. Boia hauen jabegoa eta mantenua, Bilbao-Vizcaya eta Matxitxako deitutakoak, Puertos del Estado-rena eta Euskalmet-ena da, hurrenez hurren. a-klorofilaren kontzentrazioa (fitoplankton biomasaren adierazle gisa) ur zutabean bertan neurtu da Sea-Bird CTD (Conductivity, Temperature and Depth) baten bitartez.

Itsaso zabaleko laginketa-estazioan urtaro hotzetan biziraupen tasan

efektu nagusia baldintza ozeanografikoek dute. Hala ere, dentsitatean topatutako aldakortasun handiak aditzera ematen du, olatuek ez ezik, bestelako kanpoko faktoreek, hala nola hazien kolonizazioak, harrapari naturalak, kudeaketa akatsek edota biofouling kantitateak, eragina dutela urtaroka edota behin-behineko indibiduoen galeran. Litekeena da, 5m-ko sakoneran aurkitutako dentsitatearen igoera kolonizazio berri baten ondorio izatea. Hain zuzen ere, udazkenean hazi tamainako muskuiluen kopurua udaberrian guk landatutako muskuiluen kopurua baino handiago dela aurkitu dugu luzera-maiztasun datuak aztertzean. Ez da honelako kolonizaziorik aurkitu 15m-ko sakoneran.

Produkzioa estruktura pilotuan jaitsi egin da bi sakoneretan, aurkako baldintza hauetan, udazkenetik negura. Urtaro kaltegarri horietan hilkortasun tasa, kolonizatzaile berri hauengatik ezin izan da ondo neurtu 5m-ko sakoneran. 15m-ko sakoneran produkzioaren galerak (sistematik eroritako eta hildako muskuiluak) bi urtaro horietan, udazken eta neguan, nabarmenak izan dira. Produkzioaren galera %42ko izan da guztira, kontutan harturik kultibo fase guztiak, haziak landatzetik bilketaraino. Horregatik, ikertutako laginketa-estazioetan estimatutako hilkortasun tasa, muskuiluak modu oparo batean produzitzen diren herrialdeen adinakoa dela ikusi da.

Ikertutako laginketa-estazioetan hazkuntza tasa esanguratsuki

urtarokoa da. Neguan egonkortu egiten da hazkuntza tasa eta muskuiluak geldoago hazten dira. Iritsi diren tamaina mantentzen dutelarik hazkuntza geldoko urtaroan. Udaberritik udazkena bitartean hazkuntza tasa handitu egiten da, eta hazkuntza maximoa udaran aurkitu da. a-klorofilaren kontzentrazioak ziklo bimodala erakutsi du ur azaleko uretan, maila gorena udaberriaren hasieran eta udazkenaren erdialdera eman delarik, eta mailarik txikiena, berriz, udaran.

Hazkuntza positiboa eta a-klorofilaren kontzentrazio baxuak, udaran muskuiluek janari iturri ez fitoplanktonekoa erabili dutela adierazten du. Gainera, ikerketa honetan ikertutako markatzaile trofikoen gantz azidoek erakutsi dute muskuiluen dieta udaran ez dela soilik dinoflagelatu eta diatomea barietate batzuek osatuta, baita ere bakteria, partikula organiko eta landare eta animali ugariren zitolisatutako zelulen detritusak. Jateko estrategiak kontutan harturik, urtaroka estrategia ezberdinak aurkitu dira eta bildutako tamaina komertzial ezberdineko muskuiluen artean estrategia ezberdinak topatu dira.

Etorkizuneko produkzio bati dagokionez, haziak udaberri bukaeran landatzen badira (ikertutako kasuan bezala), soken mehetze prozesu bat udara amaieran edo udazken aldera egiteak dentsitatea kontrolatzen lagundu ahalko luke. Modu honetan “kendutako biomasa” minimizatu ahalko litzateke. Bestalde, sokak kolonizadore berriez garbitu ahalko

lirateke, guk landatutako muskuilu sortarekin errekurtsoengatik eta sokan lekuarengatik lehiatuko litzatekeena. Soken mehetze prozesua neguan egin liteke soketako populazioaren %42a tamaina komertzial txikikoak direla ikusi bai da. Soketan tamainaren arabera muskuiluak sailkatzeak hazkuntza sustatuko luke eta baita, in situ galerak txikitu ere.

Ikertutako eremuan, muskuiluak biltzeko garairik oparoena udaberrian landatutako muskuiluekin, hurrengo udara litzateke. Hala ere, muskuiluen bilketa udara amaieratik udazkenerarte atzeratu beharko litzateke errute garaiak saihesteko. Bilketa merkatuko eskaria asetzeko (neguan) garai ez optimo (udara) batean burutzearen arazoa arintzeko, salmenta urte guztian zehar bermatuko duen urte osoko prozesaketa-instalazioak egin lirateke. Berez, ikertutako estaziotan estimatutako handitze funtzioa argi eta garbi sasoikoa izateak, urte guztian zehar muskuiluak biltzea posible dela frogatzen du. Ikertutako laginketa-estazioetan muskuilu hazien landaketarako garairik oparoena udazkenaren hasiera litzateke (irailean), eta kasu horretan, bilketa optimoa hurrengo udazkenaren hasieratik neguaren amaierararte (i.e., hazkuntza geldoko sasoian) egin ahalko litzateke. Hortaz, ikertutako laginketa-estazioetan kultibo denbora tamaina komertzial handiko muskuiluak (>70 mm) ekoizteko urtebetekoa litzateke, landatzea aipatutako garai optimoan egingo balitz eta bestela, kultiboak urte

eta erdi iraungo luke, landaketa garai ez optimo batean egingo balitz (ikertutako kasuan bezala).

Muskuiluek tamaina komertziala lortzen dutenean eta hazkuntza geldoko sasoira iristen direnean, edozein momentutan bildu ahalko lirateke urtaro horren barnean. Gainera hazkuntza geldoko sasoian bilketaren kudeaketa, arrazoi logistikoei jarraituz egin ahalko litzateke. Hala ere, muskuiluen baldintza urtaroka aldatzen da eta hazkuntza geldoko sasoian, muskuiluek baldintza baxuagoak izan ditzakete eta hortaz, produktu finalaren kalitatea aldatu daiteke. Kalitate ezberdineko muskuilu produktu hauek merkatu ezberdinetara bideratu daitezke (e.g., kontserbagintza, freskoa).

Kultibo sisteman bi sakoneren artean 10 metroko aldeak, produktu finalaren kalitatean efektu garrantzitsurik sortzen duen froga argirik ez da aurkitu. Itsaso zabaleko laginketa-estazio honetan sakonera maila ez omen da nahikoa sakonera ezberdinetako muskuilu produktuaren artean alde nabaria sortzeko. Hortaz, bilketaren kudeaketa bi sakoneretan, kontsiderazio logistikoak kontutan hartuta egingo ahalko da.

Urteko ekoizpena 5m-ko sakoneran 15m-ko sakoneran baino handiagoa dela aurkitu dugu, hazkuntza tasa handiagoa, bukaerako haragi masa handiagoa eta soketako dentsitatea handiagoa delarik. Muskuiluen sailkapenean (muskuilu zenbakia kg -1) aldea aurkitu da bi

sakoneren artean. Hala ere, alde hori ez da nabarmena izan eta, beraz, arrazoi komertzialak medio bilketa lanak bi sakoneretan batera egin ahalko lirateke.

Itsas zabaleko laginketa-estazioko muskuilu esperimentalek Galizian kultibatu eta merkatuan eskuragarri dauden muskuiluek baino oskola altuagoak eta zabalagoak dituztela aurkitu da. Bi muskuilu produktuek konposizio biokimiko antzekoa erakutsi dute, naiz eta muskuilu esperimentalek errauts kantitate altuagoa duten. Gantz azidoen profila aztertzean, diferentzia estatistikoak sumatu dira. Markatzaile trofikoak diren gantz azidoak elikadura estrategia ezberdinak izan dituztela iradoki dute ere bai. Ezberdintasun hauek zentzumen analisian ez dira guztiz nabaritu. Hala ere, dastatzaileek karakteristika bereizgarriak, itsaso zapore intentsua, testura bigun eta mamitsuagoak eta zapore gaziagoak identifikatu dituzte itsaso zabaleko laginetan. Diferentzia hauek baztertuta, zentzumen analisia tamaina komertzialeko laginketa-estazioko muskuiluek eta merkatu lokalean eskuragarri diren tamaina bereko muskuiluen arteko alderaketan erabakigarriak izan dira. Bi muskuilu produktuek 7 puntu lortu dituzte kalitate kategorian eta aztertu dituzten kontsumitzaileek erosteko asmoa, % 70 baino handiagoa adierazi dute. Horregatik, itsaso zabaleko muskuiluak, orokorrean, Galiziatik Euskal Herrira inportatzen diren muskuluen antzekoak direla

esan dezakegu. Marketing perspektiba batetik, Bizkaiko Golkoaren hego-ekialdean, itsaso zabalean gizendutako muskuiluak kontsumitzaileek ondo onartuko lituzketela esan dezakegu, eta gaur egun merkatu lokalean dagoenarekin alderatuta, gutxieneko baldintzak beteko dituztela ere esan dezakegu.

Ikerketa honen emaitzak Bizkaiko Golkoaren hego-ekialdean itsaso zabaleko laginketa-estazioan muskuilu (Mytilus galloprovincialis Lmk.) kultiboari buruzko lehen datu ekologikoak eta aurretiazko lehen produkzio analisiak dira. Aurkikuntza hauek, zonalde espezifiko honetan, muskuiluen akuikultura kostaldetik kanpo mugitzera sostengatzen dute.

ABSTRACT

ABSTRACT

The level of fishing exploitation and the decrease of capture volumes in the Bay of Biscay creates the need to find alternatives that could diversify fishing activities and create employment within this primary sector community of the Basque Country. The projected human population will require a significant increase in food production and especially in animal protein. *Mytilus galloprovincialis* Lmk. is a natural choice in the SE Bay of Biscay and this work analyzes if the baseline biological conditions for a successful mussel aquaculture industry are met in the Basque Country.

Mussel culture relies firstly on the carrying capacity of the ecosystem wherein it is developed, and secondly, the success of culture will rely on the mussel species, cultivated life cycle of the animal, as well as the specific environmental and socio-economic factors wherein the activity is carried out. Space available in coastal areas for aquaculture facilities is growing more and more limited and is subjected to strict jurisdictions and limited leases. In addition, the geography of the coast in many regions might not be sheltered enough for mooring rafts or longlines, while limited seston supply and/or polluted waters can also represent limiting factors for mussel coastal aquaculture.

There are few examples of successful biological and engineering offshore submerged longline bivalve aquaculture technology (Langan,

2001; Langan and Horton, 2003; Buck, 2007). Longline systems evolved from the Japanese suspended oyster-culture technique, consisting of either surface or submerged longlines, held in place with anchors and supported by buoys and floats. In locations with adverse sea conditions, such as the present study area in the SE Bay of Biscay, submerged longlines are considered the only option.

Hence, this study investigates an interesting alternative to traditional suspended mussel culture in coastal inlets of the Basque Country. This study had two main objectives. The first was to assess the reproduction and settlement patterns of native mussel populations inhabiting the Basque coast, to determine if recruitment on the shore could supply the offshore culture system with natural seeds. The second objective was to assess growth, survival and product quality of the mussel *Mytilus galloprovincialis* Lmk. cultured in the open water of the SE Bay of Biscay in a pilot culture system at two culture depth scenarios, to determine if conditions fulfill market requirements for a sustainable and profitable aquaculture production. A secondary objective of this study is to assess the causal relationship among environmental factors modulating those biological performances in both inshore and offshore study areas in order improve culture management.

The work is divided in VII chapters. In Chapter I, a general

introduction of open water aquaculture (historic facts, origin, pros and cons) as well as the general aspects of mussel biology are presented. Also in this chapter, our objectives and tested hypothesis are presented. Chapter II describes the reproductive cycle of 5 adult mussel populations along the Basque coast, in three estuarine and two nearshore open coastal sites. Chapter III describes the settlement and recruitment pattern along the Basque coast. Chapter IV and V describe seasonal growth and biological performance of mussel *Mytilus galloprovincialis* Lmk. cultured in a submerged longline system in the SE Bay of Biscay at two culture depth scenarios. Chapter IV describes the production of mussels harvested seasonally on the culture system, as well as the quality of those mussels. Chapter V describes the real growth performance of the original seed batch (without newly settled mussels) cultured in the pilot system. This chapter analyzes the production and biomass variations. In addition, this chapter describes the quality of mussels of a commercial shell length ("small" and "large" commercial mussels, from the original batch plus newly settled mussels) harvested seasonally. Chapter VI describes the comparison regarding consumer acceptance of commercial size mussels ("large commercial mussels"; >70mm) cultured in the open ocean compared to other mussels of same size currently available in the market. Chapter VII includes conclusions of the work, suggested further

research and, finally, the hypothesis of this work is answered.

The Basque Government has no jurisdiction on seafood production in the open waters of the SE Bay of Biscay, north of the Basque coast. Owing to this, licenses from the Spanish Government were applied for in order to install a pilot submerged aquaculture system at two miles off the coast. Posterior license processing took more than one-and-a-half years. Therefore, open water culture system deployment and previously predicted culture times of the study were delayed. Nevertheless, the first part of the study held at inshore areas was repeated for a second year to overcome this delay. The second part of the study held at the offshore area was subsequently correctly executed.

The reproductive health status of *M. galloprovincialis* Lmk. from five sites, three estuarine and two nearshore open coastal waters, along the coast of the Basque Country over two consecutive years (2010 and 2011). Sex ratios, developmental stages of gonads, as well as reserve tissue cycle were analyzed. The two nearshore open coastal waters and the three estuarine sites where studied mussel populations inhabit are clearly differentiated due to salinity and alternative food sources (e.g., organic carbon). On the Basque coast seasons are clearly characterized by the temperature cycle as well as the suspended solids cycle. Finally, Chlorophyll-a peaked in the estuaries during spring and summer, while

lower peaks were found in fall and winter at the open coastal sites.

Along the Basque coast gametogenesis onset was observed from winter to spring. Spawning occurred from spring till summer. Differences in the timing of spawning were observed depending on site, and in some sites year-to-year variability was also observed. Regarding differences among sexes, only the Mutriku coast showed a significant sex-ratio bias in favor of males and only in Oiartzun estuary were the two hermaphroditic individuals of the study found. Moreover, asynchrony between sexes was observed in the timing of the reproductive cycle at these two sites. After spawning, from late summer till fall, the level of oocyte atresia increased and a corresponding increase in the reserve tissue (ADG cells) was observed. Regarding the other reserve tissue (VCT cells) their level increased in fall and in spring. Hence, it can be considered that mussels inhabiting these sites display normal reproductive cycles. Further, they could become a source of wild mussel spat for expected commercial mussel farming. In doing so, and in order to secure and protect maximum reproductive outputs, mussel harvesting along the Basque coast should be avoided from spring to the end of summer.

The annual settlement and recruitment patterns of *Mytilus galloprovincialis* Lmk. along the Basque coast were assessed simultaneously to reproductive health status study using collector ropes deployed near

aforementioned five mussel populations. This enabled the discovery of a potential relationship between observed primary settlement and the spawning period of wild mussel populations. The highest abundances of primary settlers were observed from early summer to fall. Nevertheless, recruitment of juvenile mussels on the Basque coast is more determined by post-settlement processes than by the initial settlement abundances and thus, the resultant wild mussel populations are not very large. Eastern estuaries showed higher settlement as well as higher recruitment rates than western estuaries. Nearshore open coastal waters showed intermediate primary settlement and final recruitment abundances. Differences among studied sites were attributed to differences on temperature, chlorophyll-a levels and water movements observed at both years. Due to user conflict the space for the allocation of areas for seed gathering may be limited on the Basque coast. Nevertheless, mussel seeds collected at these studied sites, particularly Bidasoa estuary, could serve to supply grow-out sites in the open ocean . Hence, this seed gathering could help to fulfill future seed demand for commercial purposes. If these sites are finally to be used, care should be taken as to when to harvest ropes. In order to better control post-settlement processes ropes could be transferred in late summer-fall to aquaculture facilities.

ABSTRACT

Regarding open ocean aquaculture, *Mytilus galloprovincialis* Lmk. juvenile mussels were sub-tidally collected from natural populations and transferred (by manual seeding) to 24 target growing ropes suspended and distributed (12:12) from the longline system at two different culture depth levels (5m and 15m depths). The initial mussel mean size and density were 17.40 ± 5.07mm and 681 ± 37 individuals per linear meter. Mussels were cultured from June 2013 to August 2014. At each sampling time, three mussel ropes from each culture depth level (5m and 15m depths) were randomly sampled every 4-5 months (depending on environmental conditions). Mussel lengths and abundances were used to estimate density (individual's·m^{-1}) and mortality on ropes, as well as to estimate the absolute size frequency observed on 36m of rope (three ropes) per sampling depth and per sampling time.

Growth performance, allometry of the shell, condition index, and biochemistry of mussel (*Mytilus galloprovincialis* Lmk.) was measured in the open water culture scenarios. Growth in weight was measured through weight-at-length allometric relation, and instantaneous (G) and Daily specific growth rate (G%) of harvested ropes were also measured. Similarly, growth in mussel length on ropes per day unit was measured using exponential relation. New recruits were observed at 5m culture depth scenario. In order to assess the real growth rate of the seeded

batch (without newly settled mussels) the measurements of shell length were grouped into 10mm length-class ranges (constant size classes) and the resulting length-frequency tables were resolved into Gaussian components, using the method of Bhattacharya (1969) with the program FISAT II. The mean length of the presumed principal age-class (the original seed batch) from these resolved modal values at each sampling time and culture depth scenario, were fitted to a von Bertalanffy seasonal growth function (VBSGF). Mussel quality characteristics (i.e. condition, biochemical and fatty acids composition) were determined seasonally at both culture scenarios. In addition, the quality characteristics of mussels of different commercial sizes ("small" and "large" commercial sizes; 50-70mm and >70 mm) were analyzed seasonally. Finally, the quality of "large commercial mussels" cultured in the open ocean was compared to other mussels of the same size currently available on the market considering condition index, biochemical and fatty acids composition analyses as well as with a consumer sensory evaluation.

Local environmental conditions on seawater temperature and currents at the experimental site were obtained from the NEMO model (Nucleus European Modelling of the Ocean). Information on waves (i.e., significant wave height, H_s, and peak period, T_p), was obtained from two metocean buoys, called Bilbao-Vizcaya and Matxitxako, owned

and maintained by Puertos del Estado and Euskalmet, respectively. Chlorophyll-a concentration (as indicator of phytoplankton biomass) was measured *in situ* in the water column by means of a Sea-Bird CTD (Conductivity, Temperature and Depth).

In the open ocean pilot system, oceanographic conditions are likely to be the major factors controlling survival success in the studied area during colder seasons. Nevertheless, the observed high variability of mussel density within the present study may indicate that not only waves and currents but also some other factors such as oversets of mussels seed, natural predators, management errors and or biofouling quantity, could have affected the seasonal or eventual loss of individuals from ropes. The increase in density at the shallower culture depth during fall was likely due to overset of mussels of seed size. Indeed, the length-frequency data showed that the number of mussels of seed size observed in fall was higher than the original number of mussels of that size seeded in spring. This settlement was not noticeable on ropes from the deeper culture depth.

The production in the pilot structure decreased at both depths under these unfavorable conditions, found from fall to winter. In those unfavorable seasons mortality could not be correctly measured at 5m depth due to the presence of aforementioned new recruits. At 15m depth,

the production losses (fall-off as well as mortality) in both seasons, fall and winter, were substantial. Nevertheless, at 15m culture depth the loss due to mortality, taking into account all culture phases from seeding to harvest, was approximately 42%. Hence, the mortality rates found at the study site may well be similar to values found at sites where mussels are successfully produced.

In the studied area mussels' rate of growth was found to be significantly seasonal. During winter growth stabilizes and mussels grow more slowly. They maintain their reached size during these slow growing months and increase thereafter from spring to fall. Maximum growth was observed in summer. Chlorophyll-a showed a bimodal cycle in surface waters, with maximum levels observed around early-spring and mid-fall and minimum levels observed in summer.

The positive growth and low chlorophyll-a concentrations found during summer may indicate the utilization of a non-phytoplanktonic food source by mussels in the studied area. In addition, fatty acids trophic markers analyzed during the study showed that mussels in summer not only feed on various species of dinoflagellates, supplemented by a variety of diatoms, but also feed on bacteria, organic particles as well as detritus consisting of particles from cytolised cells of a great variety of plants and animals. Regarding feeding strategies, different feeding strategies were

observed seasonally and also between harvested mussels of different commercial lengths.

In terms of possible future production, if seeding is carried out in late spring (as in the studied case) thinning out processes during late summer-fall could help to control density, to minimize eliminated biomass as well as to clean the ropes of new recruits that would compete for space and resources with the cultured batch. This thinning out could also be done in winter as nearly 45% of the population (at both depths) were within thinning out size ("small mussel" length; 50-70mm) in the present study. The separation of mussels by their shell length would promote growth of culture mussels and improve the production yields as well as decrease the in situ mussel losses in a possible future scenario.

In the studied area the optimum time for harvesting mussels, seeded in the previous late spring, was in the following summer. Considering this scenario, however, harvesting should be delayed to late summer-early fall to avoid spawning periods. In order to mitigate the problem of harvesting at sub-optimal time (summer) for market demand (winter), year-long processing facilities could be created which could enable sales throughout the year. Indeed, the clear seasonal growth curve observed at the studied site proves that year-round harvesting is possible. At the studied site the optimum seeding time may well be in early fall

(September), in which case, harvesting may well be optimum from the following early fall to the end of winter (i.e., in the slow growing period). Thus, in the studied site the culture period to produce mussels of "large commercial size" (>70 mm) will last a year after seeding, if the seeding is done in the aforementioned optimum time or alternatively, it will last one-and-a-half years, if seeding is done in sub-optimal time (as in the studied case).

Once mussels attain their commercial size and reach the slow growing period, they could be harvested at anytime in this period. In addition, during this slow growing period harvesting management decisions could be made based on logistic reasons. Despite this, mussel condition changes seasonally and during part of the slow growing period mussels may display slightly lower condition and thus, the quality of the final product could change. These mussel products with different quality characteristics may well be bound to different types of markets (e.g., canned, fresh).

No clear evidence in meat quality was detected on final mussel product that could suggest a significant effect on the difference of 10m between culture depths tested. Depth might not be enough to promote final differences on a mussel product at these exposed scenarios. Hence, the manner in which mussels are harvested, and at which depth, will be

based on logistical considerations rather than by quality characteristics.

The annual production of mussels at 5m depth was higher than at 15m depth, due to a higher growth rate, higher final meat weight and higher final density. Differences in mussel classification (pieces of mussel product·kg^{-1}) between both culture depths, however, were not notable and thus both culture depths could be harvested together for commercial purposes.

Experimental mussels were significantly higher and wider than mussels of the same size from Galician Rías available in the local market. Both mussel products showed similar biochemical composition, although experimental mussels showed significantly higher ash content. Regarding fatty acids profile, statistical differences were observed. Fatty acids tropic markers suggest different feeding strategies. These differences were not fully reflected in the sensory assessment. However, most panelists also identified some distinctive attributes, such as intense sea-flavor, softer and more juicy textures, and salty tastes in the open ocean samples. Despite these differences, the prescreening hedonic analysis to compare if experimental open ocean cultured mussels of commercial size were as competitive as a local market leader of the same commercial size were conclusive. Both mussel products reached 7 points out of 9 in quality category and the tested consumers also expressed an intention

to purchase both products higher than 70%. Hence, experimental open ocean mussels were similar to those routinely imported from the Galician Rías to the Basque Country. From a marketing perspective, the mussels cultured in the open ocean of the SE Bay of Biscay may well be accepted by consumers and may well fulfill the minimum requirements to fit and compete in the existing local market.

The results of the present study constitute the first available ecological data and the first preliminary production analysis on the culture of mussels (*Mytilus galloprovincialis* Lmk.) in the open ocean scenario of the Bay of Biscay. The findings support mollusk aquaculture moving off the coast at this specific location.

CHAPTER 1

GENERAL INTRODUCTION

Overfishing, stock depletion and growing human population

Capture fisheries have continued to decline over last decade (Langan and Horton, 2003) and no sustainability has been achieved yet. This could be attributed to Hardin's (1968) theory of "Tragedy of the Commons" where common resources are exploited as infinite. Here, marine fisheries have been exploited as if they were infinite resources. This has led to the current situation, where more than half of the worldwide fish stocks are either exploited, over exploited or worse, depleted. Moreover, improved capture technologies, geographical expansion of fishing activities, as well as large-scale exploitation of previously exploited species, could well cause further depletion of natural stocks.

The level of fishing exploitation and the decrease of capture volumes in the Bay of Biscay creates the need to find alternatives that could diversify fishing activities and create employment within this primary sector community of the Basque Country (DMAPTAP, 2009). The general cause of this instability and low profitability of extractive fisheries nowadays is due, mainly, to the fluctuations and the negative interaction of those factors regulating fish resources, fish market prices, and petrol costs.

In terms of food demand, the projected human population (9 billion people by 2050) (FAO, 2014), will may well require a significant increase

in food production (Duarte *et al.,* 2009) and especially in animal protein. Moreover, not only is the population expected to increase, but with it a corresponding per capita consumption of fish (Ryan, 2004). The existing world capture fishing will not be sufficient to meet this future demand (Ryan, 2004), since as at best, this type of fishing activity is expected to remain static. As Coen (1995) and Marra (2005) highlight, freshwater is a limited resource, and food production will most likely have to be placed in marine environments. In coastal areas, space is scarce and there are many users (fisheries, tourism, ecologist, etc.), which creates stake-holder conflicts for harbor and disposal, sediment extraction and disposal, fisheries, tourism, as well as coastal and environmental protection. Furthermore, all these constraints found in coastal areas will evolve (Wirtz *et al.,* 2002) and future expansion of aquaculture activities inshore could be limited by the carrying capacity of coastal environments wherein these aforementioned stake-holders coexist.

Mussel Production

Aquaculture is a seafood production sector which has seen major growth over the last few decades. Mollusk aquaculture in particular, represents more than 75% (13.9 million tons) of aquaculture worldwide, with mussel production representing approximately 13% (1.8 million tons) of its annual production (FAO, 2014). 20 European countries, led

by Spain, France, and the Netherlands, produce one third of the total global mussel aquaculture production (Buck *et al.,* 2010).

Spain, with a production near 200,000 tons per year^{-1} is the second producer worldwide and the first in Europe (FAO, 2014). 95% of this production is mainly produced in Galicia, in the north western Iberian Peninsula. The remaining 5% of mussel production is carried out along the Mediterranean coast, in two Ebro delta basins in Catalunia, Fangar and Alfacs. Mussels are also produced along the Andalucian coast, where growth in production activity is rapid (Font *et al.,* 2007; Ramón *et al.,* 2007; Tirado *et al.,* 2011).

Mussels are cultured in different suspended culture types: rafts (ropes hung from floating wooden structures in protected embayment), racks (ropes attached to grounded wooden stakes close to the shoreline) and longlines (suspended culture ropes in the sea). Despite these structural differences, the main principles of cultivation are consistent throughout the different culturing techniques. Briefly, mussels for culture are derived from natural reproduction, and resulting spats settle on collector ropes. Farmers raise these mussel seeds to a marketable size without the need to feed them.

Aquaculture: evolution

"Blue Growth" is a long-term European strategy to support sustainable economic growth in the marine and maritime sectors (European Commission, 2012) and provide high-grade protein to the world's growing population (Ryan, 2004).

Over the last 20 years, aquaculture contribution to global seafood production has increased (Langan and Horton, 2003). Indeed, between 1980 and 1990 there was rapid growth in the sector of aquaculture (Bostoc *et al.,* 2010), where the 10.8 % annual growth rate (FAO 2014) was principally due to progress in technology (more efficient rearing and harvesting techniques) (Costa Pierce 2008 a and b), policies (that aimed to mitigate disease spread) (Bushek *et al.,* 2004; Forrest *et al.,* 2009) and social awareness of sustainability (mainly from growing social awareness of the ecological benefits of shellfish production for society) (Coen *et al.,* 2007). Despite this rapid growth in the 1980s, aquaculture growth stagnated in the 1990s and remained at 9.5% between 1990 and 2000. (FAO, 2014). This is due to a variety of factors including competitiveness between producers, or even single-year figures of disease or one-off environmental disasters in aquaculture production sites (i.e., observed during 2007 in Thailand, in Spain or in Canada) (Bostoc *et al.,* 2010).

Nowadays, aquaculture is a fast-growing animal food production

sector worldwide, which in 2012 accounted for 46% of the world's food fish production per human consumption (FAO, 2014). Furthermore, farming in open-ocean waters has been identified worldwide as a potential option for increasing seafood production (Bridger and Costa-Pierce, 2003). Similarly, Troel *et al.* (2009) described offshore aquaculture as a large-scale promising expansion opportunity for researchers, industry, and policy. At this juncture of offshore aquaculture, mussels are considered worldwide as attractive candidates for open-ocean farming (Hickman, 1992; Langan, 2000; Naylor *et al.,* 2000; Gibbs, 2004; Buck *et al.,* 2005; Buck, 2007). They have a fast growth rate, and provide year-round nutritious food much in demand (Seed and Suchanek, 1992; Gosling, 2003; Buck *et al.* 2010). Indeed, nowadays, the volume of global mussel aquaculture is equivalent to that of salmonid aquaculture (FAO, 2014).

Aquaculture: offshore

Offshore aquaculture (Polle, 1996; Hesley 1997; Stickney, 1998, Bridger and Costa Pierce, 2003), is an emerging scientific field for cultivating seafood in offshore sites. There are few examples of successful biological and engineering offshore submerged longline bivalve aquaculture technology (Langan, 2001; Langan and Horton, 2003; Buck, 2007). Longline systems evolved from the Japanese suspended oyster-

culture technique, consisting of either surface or submerged longlines, held in place with anchors and supported by buoys and floats (Langan and Horton, 2003). Outside of Europe, the longline system has been the most popular method for *Mytilus* cultivation (Polk, 1996; Hesley, 1997; Bridge and Costa-Pierce, 2003). Indeed, in locations with adverse sea conditions, submerged longlines are considered the only option.

In these offshore sites, culture systems are more exposed to all kinds of harsh oceanographic conditions (Ryan, 2004) than in traditional protected aquaculture production sites, such as basins and estuaries. The open ocean lacks shelter from topographical features which can mitigate the force of the ocean, as well as wind-generated waves.

Regarding distance from the shore, Ryan (2004) defined offshore aquaculture to be situated at > 1.3 nautical miles (nmi) within the continental shelf, which indeed does not present a navigational hazard and also offers easy access to harvesting sites. The average water-current speed should lie between 0.1 and 0.5m per second according to Petrell and Jones (2000) and Reidy *et al.* (2000). Langan and Horton (2002) categorize open-ocean aquaculture to be located in deep water (>50m), fully exposed to wind, as well as waves from all directions, thereby increasing vulnerability to severe storms and to significant wave heights of 9m. Buck (2007), however, established offshore aquaculture to be

located at >6nmi distance from shore, avoiding stake-holders' conflicts from near coastal areas in Germany. The south Bay of Biscay has a narrow continental shelf and 50m depth is achieved at 2nmi distance from shore. Hence, the present pilot site is a viable option as an offshore production site.

Aquaculture: offshore longline production pros and cons

Although longlines better withstand the rigors of waves and currents than the aforementioned techniques for mussel cultivation (i.e., rafts and racks) (FAO 2014), the location of offshore structures brings about inherent challenges, both to the structure itself and also to guarantee the achievement of the desired final product. Firstly, offshore aquaculture has its challenges in relation to the cost required for technical installation, which includes sophisticated and expensive mooring collocation. Adding to these financial challenges, the offshore structure must resist harsh climate and hydrographic conditions such as high wind speed, high waves, strong currents, severe storms, among others. Such conditions cause stress on the material of the structure and will affect the life-span of system units (Langan, 2001; Buck, 2007). Thirdly, special culture and harvesting techniques need to be developed for these operational offshore works. An example includes the lifting of culture ropes using specialized vessels with built-in cranes. Finally, and

equally important, are operational challenges including essential repairs which need to get done within the available weather window (Ryan, 2004).

Open ocean sites, due to distance from shore, produce less visual impact than nearshore sites which, indeed, in the case of submersible offshore production sites is even less so (Jenkins, 1979). Further, water temperature fluctuations are less extreme, and both temperature and salinity are more stable than in protected embayment. Moreover, offshore aquaculture shows lower impact on seabed, greater oxygen availability for animals, as well as improved waste dispersion due to the higher water exchange rate in the open ocean. In this way, the open-ocean longline culture method involves the shift of mussel production to a less competitive scenario but with better water quality conditions (Langan and Horton 2003). However, in near shore areas there is lower dispersion, particularly of pollutants, pesticides, near-surface agents, estuarine runoff, etc which can render these mussels produced inshore inadequate for human consumption (Pogoda *et al.,* 2013). As bivalves are filter feeders, they are known to recycle allocthonus organic matter (Mazzola and Sarà, 2001) and thus, may contribute to create an "environmentally clean" aquaculture (Shpigel *et al.* 1993a; Gibbs, 2004). In fact, offshore mussel production inside the EU is classified as an

ecologically sound food production system (EC 834/2007).

In addition to these advantages of offshore production, mussels cultured in aquaculture are *per se* native to their production site and there is no need to feed them. In addition, this open-ocean mussel culture system itself, which is made of suspended ropes, serves to enhance spat collecting. Moreover, additional spat-collecting ropes may provide further surface attachment area for spats to settle within the culture system, further enhancing final productivity of the system. (Walter and Liebezeit, 2003). Similarly, regarding productivity, submersible longline systems is also better protected against predation of seals and diving birds, although if the system is not well protected relative fish predation could be expected (Ryan, 2004).

Aquaculture: future challenges of offshore aquaculture

With the inevitability of increased food demand in the future, the development of sustainable food production technologies is imperative. Sach (2007) highlight the need to find a balance between maintaining the living standards and inherent food requirements of local populations, while minimizing resulting environmental impact. Martínez *et al.* (2007) and Costa Pierce (2008 a) recognize that future bivalve aquaculture will compete for the use of coastal resources. Indeed, Byron *et al.* (2011) and Pogoda *et al.* (2013) point to the importance of preventing socio-

economic conflict in coastal areas where demand for space is high but limited.

Mussel culture firstly relies on the carrying capacity of the ecosystem wherein it is developed, and secondly, the success of culture will rely on the mussel species, cultivated life cycle of the animal, as well as the specific environment and socio-economic factors wherein the activity is carried out (Hickman, 1992; Gosling, 2003; Buck, 2007). Soto *et al.* (2008) defined the "Ecosystem Approach to Aquaculture", as a strategy where aquaculture production is considered within the ecosystem wherein it is present. Similarly, Inglis *et al.,* (2000) and McKindsey (2006), defined bivalve mariculture's carrying capacity along four functional categories: physical (physical available space), production (stocking density), ecological (unacceptable ecological impact created by a stocking or farm density) and social (development that creates social refuse). Therefore, ecosystem carrying capacity should consider the interdependence of all culture steps of mussel production, from seed collection to operation, as well as harvesting and processing activities, and regard all of them as a whole (Mckindsey, 2006). Herein, this resultant sustainable aquaculture development will interlink between environmental and social needs and thus, will bring social equity and resilience of the natural system. This holistic approach for each unique particular ecosystem, also named

"Integrated Coastal Zone Management" (ICZM), takes into account the simultaneous consideration of the bioenergetics and the cost-benefits of all stake-holders, which is essential for future aquaculture management (Sarà and Mazzola, 2004; Ryan, 2004; Buck, 2007).

Moreover, sustainable and environmentally friendly food production is highly valued, socially. Indeed, 86% of consumers surveyed in a study, held in 2005 with European consumers from UK, Spain, and Germany, showed higher purchase intention for environmentally friendly labeled seafood (Sea food Choices Alliance, 2005). Hence, this proofs that consumers will coerce in near future an environmentally sound seafood production.

Finally, we should not forget as pointed out by many researches (Polk, 1996; Hesley, 1997; Bridge and Costa-Pierce, 2003; Buck, 2007) that in some states offshore aquaculture is beyond coastal state's control, and thus, this production is carried out without any environmental control policies (Dalton, 2004). In this juncture, there is a need to include this offshore food production in national legislations. Nevertheless, as pointed out by Ryan (2004) there is no doubt that offshore aquaculture will bring socio-economic benefits to the coastal communities where the fish food come ashore, as it will not only bring health benefits to consumers but also it would provide a valuable source of protein to a

wider population.

Regarding to this future open-ocean production, Buck (2007) pointed out that cultured candidates should require modest service needs and further, Muir (2000) pointed that future offshore aquaculture should overcome the physiological dependence on human based management through automatic monitoring, control, as well as management of the culture system.

Overview on mussels' biology

Mussel Systematic

Phylum Mollusca Cuvier (1795)

Class Bivalvia Linnaeus (1758)

Orden Mytiloide Férussac 1822

Family Mytilidae Rafinesque (1815)

Genus *Mytilus* Linnaeus (1758)

Mytilus galloprovincialis Lamarck (1819)

Mussel distribution

Mytilus galloprovincialis Lmk, is endemic and widely distributed in the Mediterranean Sea, the Adriatic Sea and the Atlantic ocean from Ireland to Morocco (Gosling, 1992; Martínez-Pita *et al.,* 2012). SanJuan *et al.* (1990), SanJuan *et al.* (1994), Marigomez *et al.* (2007) and Garmendia *et al.* (2011) described mussel inhabiting south Bay of Biscay as *Mytilus galloprovincialis* Lmk.

Nonetheless, it is worth noting that previous works on mussel along the Bay of Biscay were always referencing *Mytilus edulis* L and *Mytilus galloprovincialis* Lmk, as the two predominant species.

Mussel reproduction

Mytilus galloprovincialis Lmk. is a gonochoristic (separate sex) specie, with no apparent sign of sexual dimorphism and usually has a sex ratio 1:1 (Lubet, 1959) with a low frequency of hermaphroditic individuals (Lubet 1959; Newell, 1989; Villalba, 1995, Cáceres-Martínez and Figueras, 1998).

Genders are only distinguishable by color as spawning approaches, when the flesh of females is pale orange but whitish in males. Individuals can mature in their first 6 months or first year depending on latitude (Dare, 1983; Figueras, 1989, 1990). The size of the mussel at which maturation occurs depends on the animal's growth rate (Bayne, 1976). Bermejo (2009) pointed out that this maturity corresponds to sizes between 15 to 35 mm length in south western Bay of Biscai (i.e., Galician Rías). Young mussels spend most of their energy on growth (somatic and shell growth). Older mussels, however, spend progressively more energy on gonad development, consuming up to >90% of their energy (Thompson, 1984).

The reproductive period may be characterized by a series of gametogenic cycles, each followed by a spawning, which may occupy only a relatively short part of the entire cycle. Six main gamete developmental stages can be distinguished in mussel gonads (Kim *et al.,*

2006). Such stages include the resting stage, the early gametogenic stage, the advanced gametogenic stage, that mature stage, the spawning stage and the post spawning stage.

Mature mussels release up to 8 million eggs of 70μm diameter (Bayne *et al.,* 1978; Widdows, 1991) into the environment, and external fertilization (Strathmann, 1987) takes place when water temperatures reach 10 ^{o}C (Rutherford, 1994). Although temperature is the principal factor controlling broader aspects of the annual cycle (Seed, 1976), the duration of the spawning stage is more variable, and seems to be controlled by the nutritional condition of the animal and the animal's fecundity (Seed, 1976; Newell *et al.,* 1982; Lowe *et al.,* 1982; Seed and Suchanek, 1992; Cartier *et al.,* 2004).

Several authors have suggested that annual variation in reproduction is related also to the existing food availability of the environment (Cartier *et al.,* 2004; Dridi *et al.,* 2007). Two spawning patterns are found in literature for *Mytilus* sp.:

- Gonad development in fall-winter and spawning in spring-early summer.
- Gonad development being delayed till spring with spawning taking place in late summer.

Both patterns are related to food availability in the environment.

The first pattern is related to high food availability in summer and gametogenesis progressing in fall-winter, while the second pattern is related to low food availability in summer with gametogenesis being delayed till spring.

Studies held in the south eastern Bay of Biscay found gametogenesis proceeding in winter months and active reproductive periods occurring in spring and/ or fall (thus, with one or two spawning peaks per year in geographically close sites or in the same sites in different years) (Ortiz-Zarragoitia *et al.,* 2011; Ortiz-Zarragoitia and Cajaraville, 2010; Garmendia *et al.,* 2010; Cuevas *et al.,* 2015). These results are consistent with the notion that there are differences in gonad cycles among mussel populations in temperate latitudes. Indeed, as consequence of the seasonality of environmental conditions in temperate latitudes, mussels exhibit cyclic changes in their reproductive stages (Cartier *et al.,* 2004; Prato *et al.,* 2010).

Mussel recruitment

24 hours after fertilization cilia appears and within two days trochophore larvae stage is reached. The larva soon develops (within one to four weeks) to veliger larvae-stage when a shell is fully formed. There is little agreement to the time of larval development in pelagic

stage. According to Widdows (1991) larval development takes between 3 weeks and 3 months, according to Seed (1969a) 3 to 5 weeks, according to Fuentes *et al.* (1998) 4 to 6 weeks, according to Cáceres-Martínez (1994) 4 to 8 weeks and according to Aguirre (1979) 7 weeks.

When the larva is approximately 150μm in length, the umbo appears. Then, at 210 μm length, the extensible foot and the "eye" appears (Lutz *et al.,* 1980) and thus, the pediveliger larvae stage is reached. This pediveliger larva is ready for metamorphosis into a young mussel or spat.

Pediveliger larvae are able to attach themselves through byssal threads onto the substratum (Seed, 1969a; Bayne, 1976; Widdows 1991). Permanent settlement occurs when adequate substratum is found (larvae can delay metamorphosis for several weeks if adequate substratum is not found; Seed, 1969a) and a sessile and gregarious lifestyle starts (Bayne, 1964; Cáceres-Martinez *et al.,* 1993a).

Thus, this settlement process links pelagic and benthic stages (Merge, 1992) as it involves the initial attachment of larvae to the substratum and can significantly influence the final distribution and abundance of adult mussel populations (Bownes, 2009). The variability of mussel settlement relies on the timing and magnitude of larval supplies in addition to the physical conditions found by the animal in its environment.

The term "recruitment" refers to the number of larvae that have

settle and survived for a certain period of time including a period of post-settlement mortality (Keough and Downes, 1982). This post-settlement mortality may be the cause of large-scale differences in the composition and structure of the mussel communities inhabiting the shore, and may obscure previous settlement patterns (Osman and Whitlatch, 2004).

Small mussel populations can be found along the whole coastal length of the Basque Country. However, on this Basque coast the spatial and temporal dynamics of mussel spat arrival to these mussel beds inhabiting the coast are highly variable and poorly understood.

The period of maximum spat settlement in the south western Bay of Biscay (i.e., Galician Rías) is between May and September (Cáceres-Martinez 1993a). Some authors (Andreu, 1958; Aguirre, 1979; Mariño *et al.,* 1982) described a second spawning of mature mussels during fall, which, due to low temperatures may result in a low or nearly null spatfall. Nevertheless, Cáceres-Martínez *et al.* (1993a) described a unique spatfall occurring in spring and a weak increase of spatfalls in fall, possibly related to mussel seed migration (bysus threads break due to water movement and/or due to unsuitable substrate) and not from a new spatfall.

A secure supply of spats is a basic requirement for mussel culture and thus, naturally rich areas of mussel spat have been used in the past in large culture operations around the world (Mason, 1976; Hickman, 1992;

Langan and Horton, 2003). Mussel seeds for culture can be collected either from natural mussel beds inhabiting the shore or alternatively, with artificial collectors, such as hanging ropes. Although different techniques and materials can be deployed for spat capture, the timing and location of deployment appear to be the most important factors (Langan, 2000).

Mussel growth and condition

In mussel culturing seed size mussels (i.e., 15-25 mm) are bound to ropes with degradable cotton sleeve and these ropes are attached to the horizontal head rope of the longline (Jeffs *et al.,* 1999; Langan and Horton, 2003).

Growth in marine bivalves is principally affected by seawater temperature, food availability, and salinity (Hrs-Brenko and Calabrese, 1969;Seed, 1976; Widdows *et al.,* 1979; Rodhouse *et al.,* 1984; Page and Hubbard, 1987; Seed and Suchanek 1992) as well as exposure to air and genotypic characteristics (Mallet and Carver, 1989). Other factors affecting growth rate are related to culture system and include variables such as depth, position of ropes along the longline, and the position of bivalves relative to the water current. Steffani and Branch (2003) suggested that mussels grow faster at exposed sites than sheltered sites,

as food availability is higher in sites with greater water flow. The main difficulty in finding a causal relationship between environmental factors on growth rate is that these factors may co-vary and apparent correlation among those factors and growth may not in fact be causal (Richardson *et al.,* 1990).

Bayne (1965) explained *Mytilus* growth to be linear for larval lengths and asymptotic for adults, and Seed (1976) described high variability on individual growth rate within populations. Grow-out time on culture systems to reach marketable size is site dependent and highly variable. The grow-out time of mussels, from seed size of 20 mm length, can range from 8 up to 18 months (Langan, 2001). In general terms, mussel growth seems to be seasonal, with gross growth concentrated in one season (Seed, 1973). Shell growth, however, appears to be continuous during the year (Stirling and Okumus, 1995).

Regarding temperature, a non-linear relation between temperature and growth can be expected when the range of temperatures are too wide (Ramón *et al.,* 2007). Almada-Viella (1982) found that the growth rate of *Mytilus edulis* increases logarithmically between 3ºC and 20ºC, but declines sharply above 20 ºC. In addition, 25ºC was considered by Caciun (1980) as the upper temperature limit for *Mytilus galloprovincialis* (Lmk).

Shell length, height, and width are influenced by variation in size and shape (Thippeswamy and Joseph, 1992). Factors affecting shell shape morphology are density, age, wave exposure, mussel size and available food (Seed 1973; Akester and Martel, 2000; Alunno-Bruscia *et al.,* 2001).

The condition index (CI) relates the flesh weight to the amount of shell, which shows the relative resource allocation to tissue or shell growth in the animal, and thus could be used as a measure for physiological status of the individual and/ or population (Seed and Suchanek, 1992; Steffani and Branch, 2003). As the flesh weight includes gonad tissue, temporal changes in condition index are related to spawning events rather than food availability (Steffani and Branch, 2003). Nevertheless, CI may also be adversely affected to stress episodes (food availability, intense intra-specific competition among population, etc) that cause weight loss of flesh due to the mobilization of energy reserves (Bayne, 1965; Dame, 1996). Hence, this parameter could serve to evaluate the physiological state, health and commercial quality of mussels (Dridi *et al.,* 2007).

Mussel filter feeding

Thompson (1984) stated that food availability is the most important factor modulating growth. Tissue production relies on food availability since mussels are sessile suspension feeders (Bayne, 1965; Hawkins

and Bayne, 1992). The energy ingested as food must exceed the cost of metabolic maintenance in order for growth and reproduction to take place. When energy intake is less than the metabolic cost of maintenance, this use of bodily reserves results in negative flesh growth. The surplus energy product of the absorbed ration when maintenance metabolic cost is met is used to generate byssus, shell and body tissue, including gonads (Hawkins and Bayne, 1992).

Phytoplankton is the principal diet compound for mussels found among seston (Rodhouse, 1984b; Smaal and van Stralen, 1990), but they can also use non-phytoplanktonic carbon to meet their requirements (even up to half of the requirement) (Héralt, 1987; Newell *et al.,* 1989; Langdon and Newell, 1990; Navarro, 2003). According to Figueras (1989), individuals can filter between 50 to 120 liters of water per day. Héralt (1987) suggested that the retention of different size range particles is not constant in mussels and that, indeed, it would rely on seston load of the system and not the animal. Mussel can filter particles of 3-5 μm Ø according to Jorgensen (1975), whereas according to Dardinac-Corbeil (1990) mussels can filter particles of 1 μm Ø. There are different studies suggesting that mussels are able to select living particles such as phytoplankton and that the excess of filtered material could be rejected as "pseudo feces" by the animal (Thompson and Bayne, 1974; Widdows

1978 a and b). Chemical composition, shape as well as the size of particles seem to be important as selection criteria (Newell and Jordan, 1996). Bayne *et al.* (1993) suggested that mussels have two strategies to maximize the use of the available POM (particulate organic matter) of the environment. When there is high SPM (suspended particulate matter) in the environment, mussels choose the high organic particulate matter and the inorganic matter filtered within is packed in mucus and rejected as "pseudo feces" (Iglesias *et al.,* 1996). The second strategy is related to a low SPM in the environment. Therefore, mussel do not select particles. Particles with high organic content are absorbed and inorganic particles are passed through the digestive system but without being absorbed (Prins *et al.,* 1991).

Mussels and human health

It is worth noting that as marine mussels are widely distributed, abundant, stationary and easy to obtain, they have been used intensively in marine science (i.e., monitoring programs for environmental protection or status assessment) and nowadays are the most investigated organism worldwide (Gosling, 1992).

The Mediterranean mussel has been a part of the human diet and medicine since classical texts of Greek antiquity (Voltsiadou *et al.,* 2010).

Mussels are appreciated for their positive effects on human health as well as organoleptic properties (Grienke *et al.,* 2014). Mussel product quality, and apparent health, is regulated by biochemical composition (water, proteins, lipids, minerals, glycogen content and minor components of hydrophilic/ lipophilic nature) (Orban *et al.,* 2002; Filgueira *et al.,* 2006; Fuentes *et al.,* 2009).

Mussels are an important food source, providing high-quality protein, minerals, essential trace elements, fat-soluble vitamins (i.e., vitamin D) as well as essential fatty acids (James *et al.,* 2013). This biochemical composition of mussels is affected by water temperature, nutrient availability and the reproductive cycle of mussels (*et al.,* 2007) and shows seasonal changes (De Zwaan and Zandee, 1972; Dare and Edwards, 1975). Ruano *et al.* (2012) noted that major differences in biochemical composition relied on: (i) feeding behavior, in terms of filtration capacity and clearance rate (Vilela 1950; Héral *et al.,* 1980; (ii) the quality and quantity of seston available in the environment; and (iii) the state of sexual maturity of the individual (Fernández-Reiriz *et al.,* 2007).

Regarding lipids, mussels have a low fat content. Nevertheless, this fat content is rich in essential fatty acids, particularly long-chain polyunsaturated variety, referred to as LC- PUFAs (James *et al.,* 2013),

and thus, mussels are a good food source for human health. There are two kinds of LC-PUFAS, both of which are important for humans. The essential ω-6 PUFAs can be obtained from plants, whereas the dietary source of ω-3 PUFAs (i.e., omega-3 "good oils") can only be obtained from seafood (James *et al.,* 2013), such as bivalves. As chain elongation in humans is too inefficient to provide energy for metabolism requirements, these LC-PUFAS need to be ingested through diet. There are several health benefits related to ω-3 intake including reduced risk of cardiovascular diseases (Kris-Etherton *et al.,* 2002; Givens and Gibbs, 2008), elimination of inflammatory conditions such as rheumatoid and osteoarthritis (Gibson and Gibson, 1998), asthma (Emelyanov *et al.,* 2002), reduction of age-related decline in cognitive function (van Gelder *et al.,* 2007), reduced risk of suboptimal neurodevelopment in the offspring of women, among others (James *et al.,* 2013). Hence the analysis of these essential fatty acids may help to assess mussel quality. Moreover, the analysis of the amount and composition of some fatty acids is also used to assess the preferred diet of the animal in the studied environment, as the presence and/or the ratio of some fatty acids can be used as trophic markers.

Such precise knowledge on biochemical composition, are an important tool for mussel culture management and commercialization.

Different production sites, with different conditions and culture technologies (e.g., open ocean submerged longline systems), could cause changes in the nutritional composition of mussel individuals, which will in turn reflect a differentiated quality as evaluated by consumers (Oliveira *et al.,* 2015). Thus, as reported by Fuentes *et al.,* (2009) those characteristics of mussel quality are origin dependent.

Quality was described by Huss (1988) as aesthetic appearance and freshness, both of which include external characteristics (appearance, feel), internal characteristics (taste, aroma and texture) as well as technical characteristics (nutrition and safety) (Bett, 1997). Hence, mussel product quality is assessed by the consumer as the result of not only chemical and biological characteristics, but also organoleptic properties, such as the appearance of the muscle, the intrinsic flavor of the flesh and the absence of undesirable components (Vernocchi *et al.,* 2007).

Mussel related legal aspects.

The most common reason for bans on mussel harvesting is the presence of toxic algae (e.g., DSP (Diarrheic Shellfish Poisoning), which severely limits mussel production. The presence of these toxic algae has increased in recent years (Torgersen *et al.,* 2005).

Moreover, EU regulation (EC) 854/2004 also needs to be considered,

and within which, bivalve production areas are classified in relation to an index of global microbial contamination (Ruano *et al.,* 2012). For instance, the production in a Class A area for commercial mussels is not subjected to depuration requirements. Guyader *et al.* (2000), Formiga-Cruz *et al.* (2002) and Romalde *et al.* (2002) pointed out that different approaches to these safety regulations should be adopted according to site (inshore, offshore), as well as to cultivation method (raft, racks, longline). Finally, as pointed out by Brenner *et al.* (2014) neither official supervision responsibilities nor legal requirements have been developed yet by any state member of the European Union with the aim of finding balanced solutions, regarding farming, processing and consumer safety in offshore aquaculture.

The European Union has a registration policy for food products, including seafood. These foods are recognized as products with Protected Designation of Origin (PDO). Regarding mussels, French mussels from Mont St Michel Bay were the first awarded with this quality mark in 2006 (Sturrok *et al.,* 2008). Galician mussels were also awarded with the same quality mark of PDO in 2008 (MAPA, Ministerio de Agricultura, 2008). Other European regions that produce mussels could also apply for this kind of quality marks.

Literature review:

Summary of studies (particularly those experiments conducted in field conditions) on reproduction, recruitment, growth, biomass and production, condition index, proximate composition and fatty acids of mussels (mainly cultivated) and other bivalves is shown in Annex I.

IMPORTANCE OF THE STUDY

Successful cultivation of any species of commercially valuable shellfish depends on the natural availability of seed, ideal environmental conditions for a rapid growth rate as well as the lack of major predators and pests (Vakily, 1989; Hickman, 1992; Rajagopal *et al.,* 1998). For effective culture and for the management of natural stocks in the shore basic knowledge of biological aspects is a prerequisite. Such knowledge includes that of the reproductive cycle of adult animals as well as settlement patterns of native mussel populations from the shore. These biological aspects should be studied on a continuous basis (Avellanal *et al.,* 2002; Suarez *et al.,* 2005). Moreover, sustained economic success can only be achieved if optimal quality products are considered seriously in terms of human consumption, considering both the needs of the market as well safety of the products.

Positive responses on biologically feasible mussel performance will help to reveal the remaining conditions (i.e., economic, socio-economic, legal, and environmental) that could promote, or not, a sustainable and profitable mussel aquaculture industry in the Basque Country.

OBJECTIVES

- The two main objectives of this study are to:

  - assess the reproduction and settlement patterns of native mussel populations inhabiting the Basque coast to determine if recruitment on the shore could supply the offshore culture system with natural seeds.

  - assess growth, survival and product quality of the mussel *Mytilus galloprovincialis* Lmk. cultured in the open water of the SE Bay of Biscay in a pilot culture system at two culture depth scenarios to determine if conditions fulfill market requirements for a sustainable and profitable aquaculture production.

- A secondary objective of this study is to:

  - assess how environmental factors modulate those biological performances in both inshore and offshore study areas in order improve culture management.

To accomplish the first objective, 5 mussel populations along the Basque coast were chosen and settlement ropes were deployed near those mussel beds. These sites, three estuarine and two nearshore open coastal sites, showed different environmental characteristics (temperature, salinity, nutrients). At these sites, mussel populations and settlement ropes were analyzed every two months over two periods of ten months each. The variability observed at these sites was of great value to understand the biological performance of wild mussel populations along the Basque coast.

To accomplish the second objective, a system was established by the regional authorities at the open ocean experimental site (43° 21.39' N, 2° 26.90' W), located at 2 miles off the coast of Mendexa (SE Bay of Biscay) and meeting the wave energy criteria described by Ryan (2004). A suspension shellfish culture system based on a conventional longline (i.e., subsurface structure consisting of anchors and submerged flotation from which mussel ropes can be suspended; Langan, 2000; Buck *et al.,* 2005) was developed as a pilot structure for the present study. The experimental grow-out rope system was built employing polyamide and poly-steel ropes suspended in the water column from horizontal headlines at submerged conditions. Grow-out ropes of identical length (12 m) were hung from two headline depths, 5m and 15m depths, at a distance of 0.5m between ropes (Figure 1). Mussel seeds were cultured for 14 months and biological performance (growth in length and in weight, as well as mussel product quality) was monitored seasonally.

To achieve both objectives, the multidisciplinary and interdisciplinary approach was needed to bring about a correct conceptual integration of knowledge, in order help to validate this culture system initiative in the Basque Country.

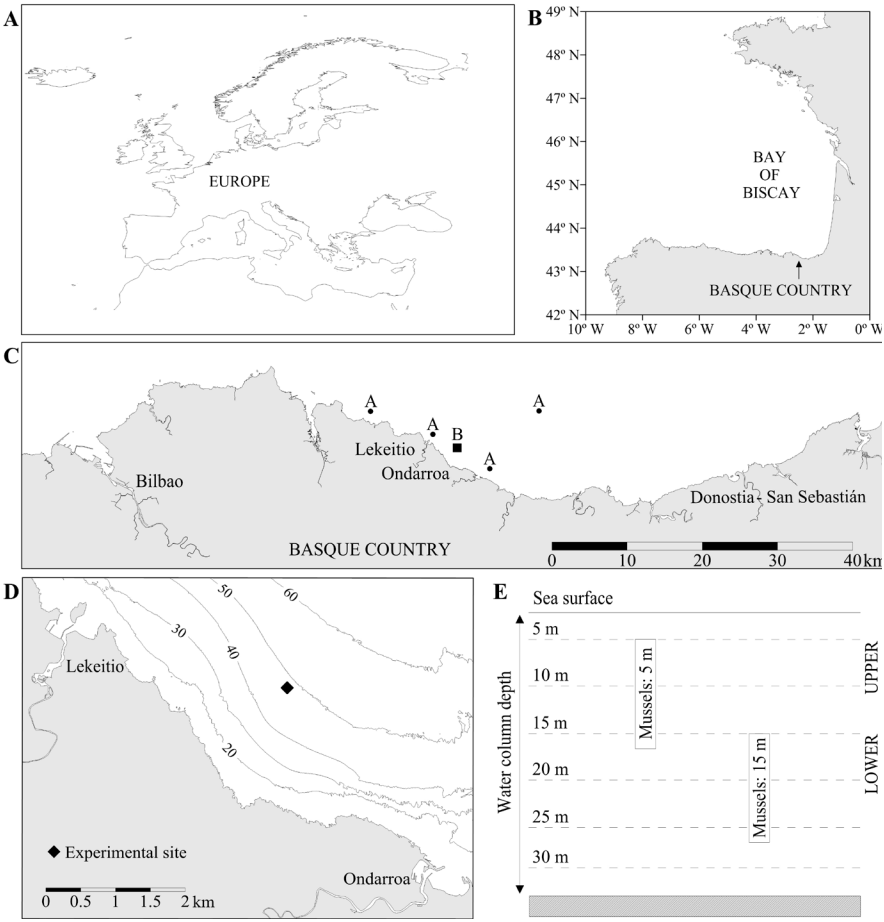

Figure 1 *A) Bay of Biscay location. B) Basque Country coast. C) Map of the study area showing the specific location of the open ocean experimental site (Mendexa: B black square) and the stations for chlorophyll-a data acquisition (A black points). D) Bathymetry map of the experimental site. E) Synthetic scheme of the suspension range of the mussel culture ropes within the water column at the beginning of the experiment.*

The following research was carried out:

- Seasonal wild population reproductive health status along the Basque coast. (CHAPTER 1).
- Seasonal seed availability along the Basque coast. (CHAPTER 2)
- Seasonal mussel condition (condition index, biochemical composition and fatty acid profile) at two culture depth scenarios in the open waters of the SE Bay of Biscay (CHAPTER 3).
- Seasonal mussel production and biomass (growth rate, survival, marketable size production), as well as commercial product quality (condition index, biochemical and fatty acid composition) at two culture depth scenarios in the open waters of the SE Bay of Biscay (CHAPTER 4).
- Mussel consumer acceptability (hedonic comparative test as well as biometrics, biochemical composition and fatty acid profile comparison) between mussels of commercial size from the open waters of SE Bay of Biscay and mussels of the same size from Galician Rías available in the local market (CHAPTER 5).

HYPOTHESIS

We hypothesize that the baseline biological conditions for a successful mussel aquaculture industry are met in the case of the Basque Country and evaluate it by means of different approaches.

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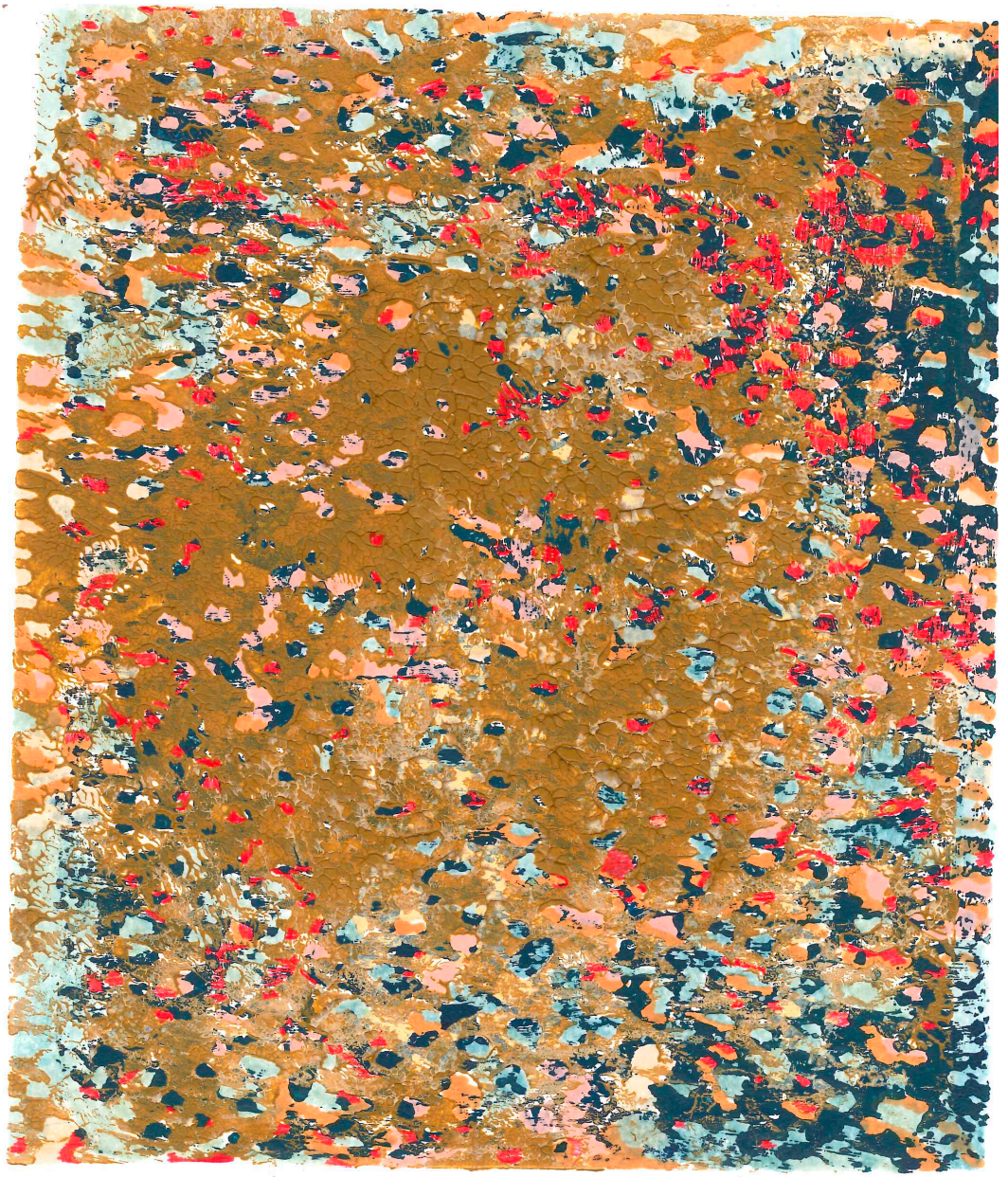


CHAPTER 2

VARIABILITY OF THE REPRODUCTIVE CYCLE IN ESTUARINE AND COASTAL POPULATIONS OF THE MUSSEL *Mytilus galloprovincialis* Lmk. FROM THE SE BAY OF BISCAY (Basque Country).

SUMMARY

Mussel commercial production depends on wild populations to obtain the seed. Therefore, any new farming initiative needs depth knowledge on reproductive cycle of native mussel populations. The reproductive cycle of M. *galloprovincialis* Lmk. from five sites, three estuarine and two coastal, along the coast of the Basque Country were assessed using Gonad Index and stereology over two consecutive years (2010 and 2011). Sex ratios, developmental stages of gonads, as well as reserve tissue cycle were analyzed. The onset of gametogenesis onset was observed from winter to spring, with spawning occurring from spring till summer. A resting phase followed with an increase in reserve tissues (ADG cell) was observed in fall. Mussels stayed ripe during winter until favorable environmental conditions were met in spring.

Differences in reproductive cycles were found among populations, and also, year-to-year variability was observed within each population. In two of the studied sites asynchrony in the timing of spawning was observed between genders. These timing differences, however, did not prevent mussels at those sites from reproducing successfully. The Bidasoa Estuary points to the potential of such studied sites for becoming the main source of wild mussel spats for future expected commercial mussel farming in the Basque Country. To ensure year-to-year maximum

reproductive output, however, wild mussels harvest should be avoided from spring to the end of summer in this region.

Key words: *Mytilus galloprovincialis* Lmk., reproductive cycle, condition index, sterology, Bay of Biscay

INTRODUCTION

Open-ocean aquaculture represents a promising alternative for sustainable cultivation of bivalve species (Polk 1996; Hesley 1997; Stickney 1998; Bridger and Costa Pierce 2003), specially in regions with limited coastal space such as the Basque Region. Analyzing the health of wild mussel populations in relation to their reproductive cycle and spat production is important as mussel production relies on the annual collection of significant amounts of wild seeds, and farmers grow these seeds in extensive marine areas (Dominguez *et al.* 2010). For commercial activity, the industry must be provided with solid information on the opportunities and risk before promoting an investment in order to produce basic aquaculture research and establish a healthy mussel industry in the region (Langan and Horton 2003). Herein, areas favorable for spat collection and/ or targeting rope deployment periods for spat settlement is of particular relevance in mussel aquaculture and further, will be fundamental for developing future farming management strategies (e.g., protect native spawning stocks and/ or larval settlement) (Gosling 1992; Dias *et al.* 2009). Moreover, prudent regulation of harvesting will maximize reproductive output of wild and cultured mussel populations.

In order to establish the adequacy of different sites of the Basque coast for spat collecting, we need to understand the basic reproduction of

wild mussel populations inhabiting those environments. The regulation of gametogenesis and spawning in marine bivalves is considered a genetically controlled response to the environment (Sastry 1979; Barber and Blake 1991). Temperature and food availability regulate gametogenic cycle and associated storage of materials (Gabbot and Bayne 1973; Seed 1976). In *Mytilus* sp. the production of mature gametes is independent of temperature and relies more on nutrient availability (Newell *et al.* 1982; Thompson 1984). In fact, gametogenesis can be energetically supported from two sources: reserves stored in the mantle tissue cells (Bayne 1976; Gabbot 1976); directly ingested nutrients (Newell *et al.* 1982; Thompson 1984); or a combination of both (Barber and Blake 1991).

The group of *Mytilus* sp. is composed of gonochoristic species with low hermaphroditic prevalence (Seed 1976). Although size for sexual maturity depends on the growth rate of each individual, sexual maturity is reached the first year of life (Bayne 1976). The reproductive system is composed of mantle tissue and consists of reproductive cells, circulatory system and connective tissue cells. The mantle tissue has two interrelated physiological functions. Firstly, the accumulation of reserves and secondly, the development of the gonad that invades the mantle proliferating at the expense of the reserve tissue.

Studies held in the SE Bay of Biscay described that gametogenesis

in wild mussels occurs in winter months and the active reproductive period in spring. In warm and nutrient rich years a second reproductive peak can be seen between summer-fall (Ortiz-Zarragoitia and Cajaraville 2010; Garmendia *et al.* 2010; Ortiz-Zarragoitia *et al.* 2011; Cuevas *et al.* 2015).

The aim of the present study was to determine and to evaluate the dynamics of the reproductive cycle in five *Mytilus galloprovincialis* propulations from the Basque Country (SE Bay of Biscay). In order to assess, both sustainable exploitation rates for this species and for estimating its potential capacity for sustainable aquaculture production, data on species reproductive cycle and nutrient storage is necessary (Mladineo *et al.* 2007). Shellfish aquaculture has been experimentally tested in the open waters of the SE Bay of Biscay (i.e., Basque Country) (43° 21.39'N; 02° 26.90'W) (Azpeitia *et al.* 2016 ; Chapter IV). As marine bivalves show a seasonal cycle of energy storage and utilization closely related to reproductive activity, the patterns of energy storage and utilization were also studied.

MATERIALS AND METHODS

Study area

The Basque coast is located in the SE Bay of Biscay. Basque region coast extends over 150 km and it is oriented E-W. There are 12 main estuaries along this coast. This study was held in 5 sites along the coast of Basque Country: in 2 coastal sites (Nerbioi and Mutriku coast), as well as in 3 estuarine sites (Nerbioi, Oiartzun, and Bidasoa estuaries) (Figure 1 and Table 1). These locations have been selected as they represent different water systems which contain large wild mussel populations along the Basque coast.

One of the studied estuaries, Bidasoa, is a shallow system (≤10 m). Also included in this study are Nerbioi and Oiartzun estuaries, whose outer zones are deeper (20-30 m). The rivers Nerbioi and Bidasoa have the highest flows along the Basque coast (26-36 m^3 s^{-1}, annual mean), whereas the river Oiartzun has a much lower annual flow (4.8 m^3 s^{-1}). Despite these differences in depth and river flow, all estuaries presented in this study have semi-diurnal tides, with tidal amplitudes that can vary between 1 m in neap tides, to more than 4.5 m in spring tides (Valencia *et al.* 2004). As such, although they present some characteristics of macrotidal estuaries, they can be considered as generally mesotidal estuaries (Hayes 1975). This study included 2 further coastal sites, Nerbioi and Mutriku

coasts, both of which are exposed and euhaline.

Environmental data

In order to characterize the environmental conditions that could influence reproduction in mussels, data on physical and chemical variables, together with chlorophyll-a concentration (as a proxy for phytoplankton biomass) were obtained in surface waters.

In the Basque Country, the environmental quality of the marine waters has been monitored since 1994 by means of the Littoral Water Quality Monitoring and Control Network (LQM) (Borja *et al.* 2004; 2009). The present study draws on data from the LQM to characterize a range of variables, including salinity, temperature, chlorophyll-a (Chl-a), total nitrogen (TN), total phosphorus (TP), total organic carbon (TOC) as well as suspended solids (SS). Environmental data are acquired on a quarterly basis (usually in February, May, August and November), in nearshore open waters as well as in estuaries. The LQM sites nearest to the biological stations were chosen for this study (Figure 1 and Table 1). Salinity, temperature and Chl-a (estimated from fluorescence) were measured "*in situ*" in the water column by means of a CTD (Sea-Bird). Laboratory methods for TN, TP, TOC, and SS can be consulted in detail in Garmendia *et al.* (2011).

Table 1 The studied systems and the geographical location of the sampling sites used for mussel populations and environmental variables. LQM: Littoral Water Quality Monitoring and Control Network.

Studied system	*Mussel population*		*LQM variables*[1]	
	Latitude	*Longitude*	*Latitude*	*Longitude*
Nerbioi coast	43.3775	-3.084	43.3845	-3.0807
Nerbioi estuary	43.3535	-3.0792	43.3623	-3.044
Mutriku coast	43.3078	-2.3808	43.3284	-2.3511
Oiartzun estuary	43.3280	-1.9229	43.3282	-1.9199
Bidasoa estuary	43.3679	-1.7915	43.3716	-1.7887

[1]*Salinity, temperature, chlorophyll-a, total nitrogen, total phosphorus, total organic carbon and suspended solids..*

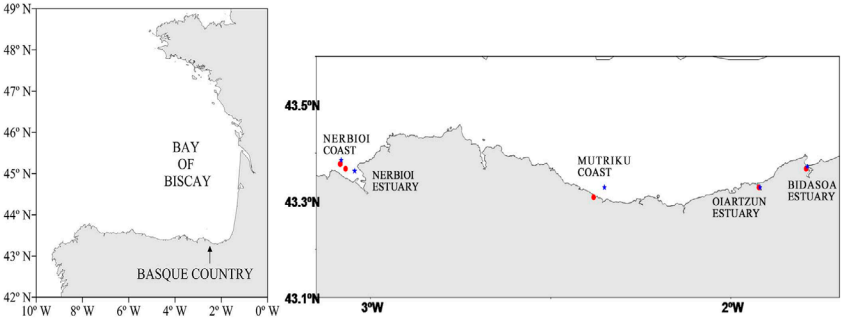


Figure 1 *Geographical location of the sampling stations used for the biological (black spots) and environmental variables analysis (crosses): Nerbioi coast, Nerbioi estuary, Mutriku coast, Oiartzun estuary and Bidasoa estuary.*

Sampling Strategy for mussels

Mussel sampling was done over two separate periods of 10 months each. The first, from April 2010 to February 2011 and the second from April 2011 to February 2012. As the reproductive cycle is influenced by seasons, samples were taken in each season: spring (April), summer (June and August), fall (October), and winter (February). As summer months are of key importance in the reproductive cycle and spat collection, two samples were taken, one at the beginning (June) and one at the end (August) of summer in both years.

At each sampling period, 20 mussels per site were randomly collected for sex and gametogenic stage determinations. Mussels were collected by hand by scuba divers during low tide at 1 m depth. Mussels with a length of 3.5-6 cm long (SE), thus sexually mature mussels (Izagirre *et al.* 2014), were collected at each site. Mussels used for histological analysis were directly processed in the field, sectioning the abductor muscle and introducing in neutral buffered formalin for 24 h.

Sex ratio, gamete development, and Gonad Index

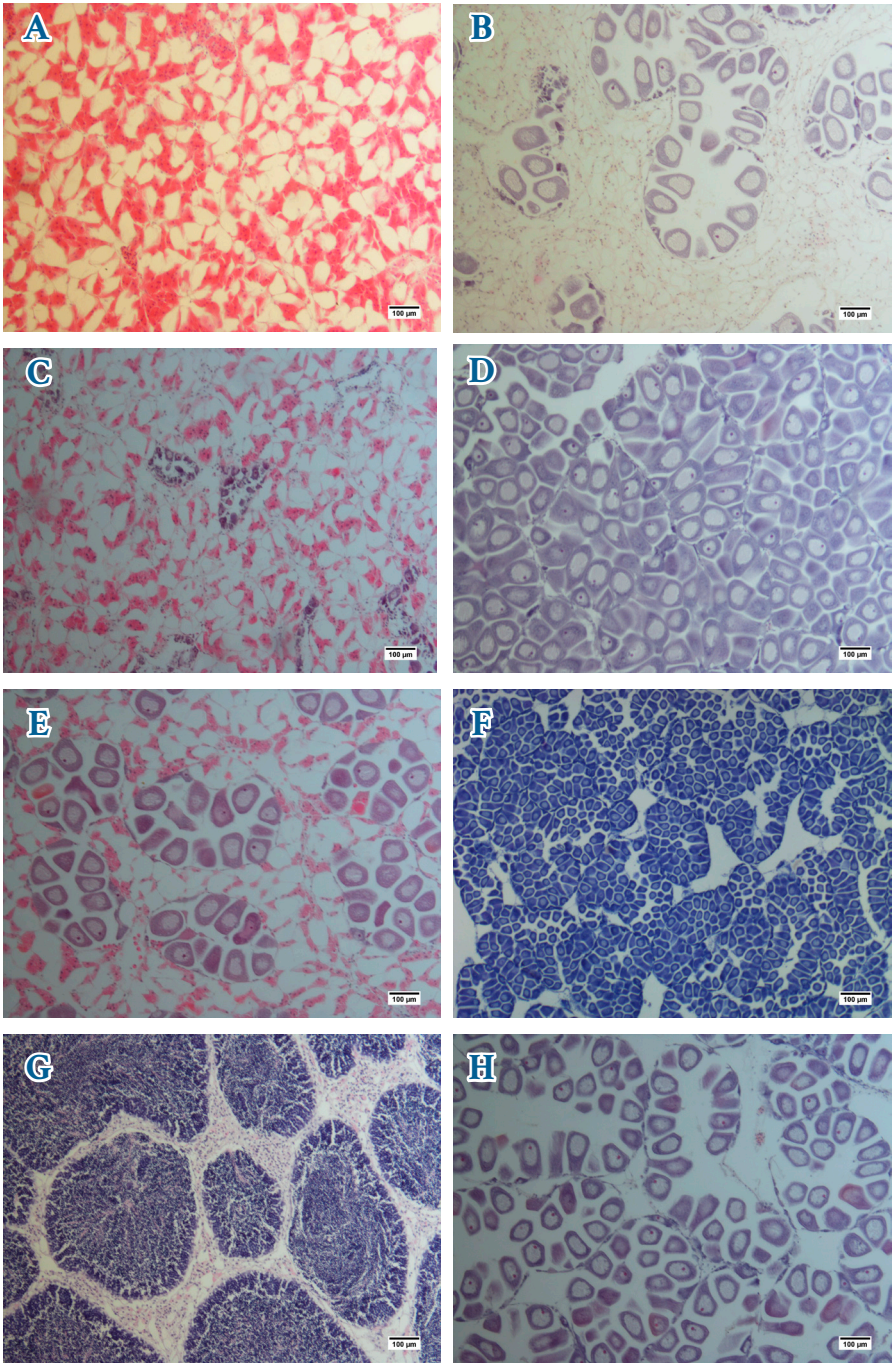
The gonad (mantle tissue) from each mussel was dissected out after fixation, placed in histological cassettes in 70º ethanol and routinely processed for paraffin embedding in a Leica ASP300 Automatic tissue

processor (Nusloch, Germany). Sections of 3-5μm thick were cut in a Leitz 1512 microtome (Vienna, Austria) and stained with hematoxylin and eosin. The slides were examined using an Olympus BX60 microscope equipped with a digital camera.

The sex of each animal was recorded to assess the total number of female and male as well as the number of hermaphrodite mussels for each site. With these data, the sex ratio (Female:Male) was calculated for each sampling/site.

The gametogenic staging established by Hillman (1984) was applied (Figure 2 and 3). Afterwards, a Gonad Index (GI) value was assigned to each development stage adapted from the description of Kim *et al.* (2006). A mean population GI value for each site and sampling time was then calculated from the sum of the individual stage numbers (Ellis *et al.* 1998). The index varies from zero, if the entire population is spent or resting, to five when fully ripped (sexually mature).

Figure 2 *Photomicrograph of female gonads from study sites. Gonad development stages in Mytilid and dreissenid, nomenclature adapted from (Seed (1975, 1976) by Hillman (1993)). 1. Resting/spent gonad (Stage 0 Inactive or undifferentiated) (**photomicrograph_A**); 2. Developing gonad: Stage 1 Gametogenesis has begun; no ripe gametes visible (**photomicrograph_ B** and **photomicrograph_C**); Stage 2 Ripe gametes present; gonad developed to about one-third of its final size (**photomicrograph_ D**); Stage 3 Gonad increased in mass to about half the fully ripe condition; each follicle contains, in area, about equal proportions of ripe and developing gametes (**photomicrograph_ E**); Stage 4 Gametogenesis still progressing, follicles contain mainly ripe gametes (**photomicrograph_F**); 3. Ripe gonad: Stage 5 Gonad fully ripe, early stages of gametogenesis rare; follicles distended with ripe gametes; ova compacted into polygonal configurations; sperm with visible tails)(**photomicrograph_ G**); 4. Spawning gonad: Stage 4 Active emission has begun; sperm density reduced; ova rounded off as pressure within follicles is reduced (**photomicrograph_ H**); Stage 3 Gonad about half empty (no photomicrograph shown); Stage 2 Gonadal area reduced; follicles about one-third full of ripe gametes (**photomicrograph_I**); Atresic gametes (**photomicrograph_ J**); apoptotic gametes (**photomicrograph_K**); Hermaphroditic gonad section from Oiartzun estuary (Spring 2010) (**photomicrograph_L**) and (Winter 2011) (**photomicrograph_M** and **photomicrograph_N**), respectively. Separate male and female follicles are observed.*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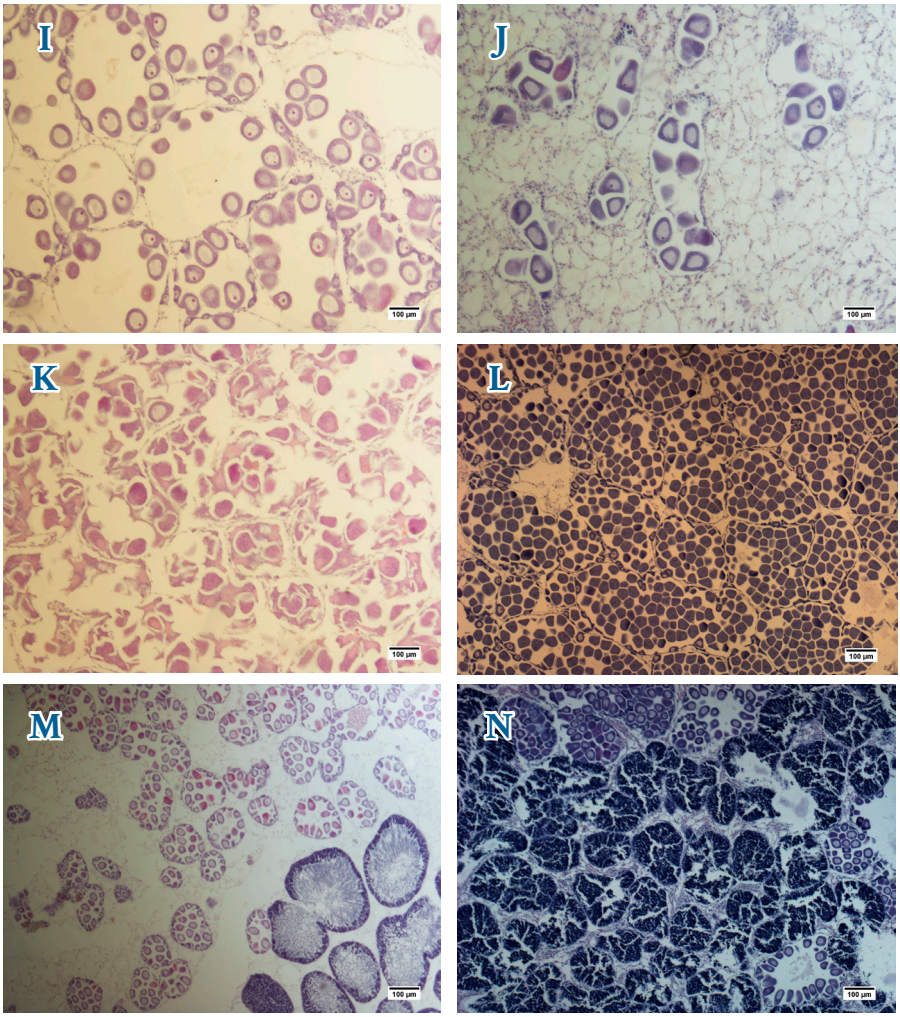
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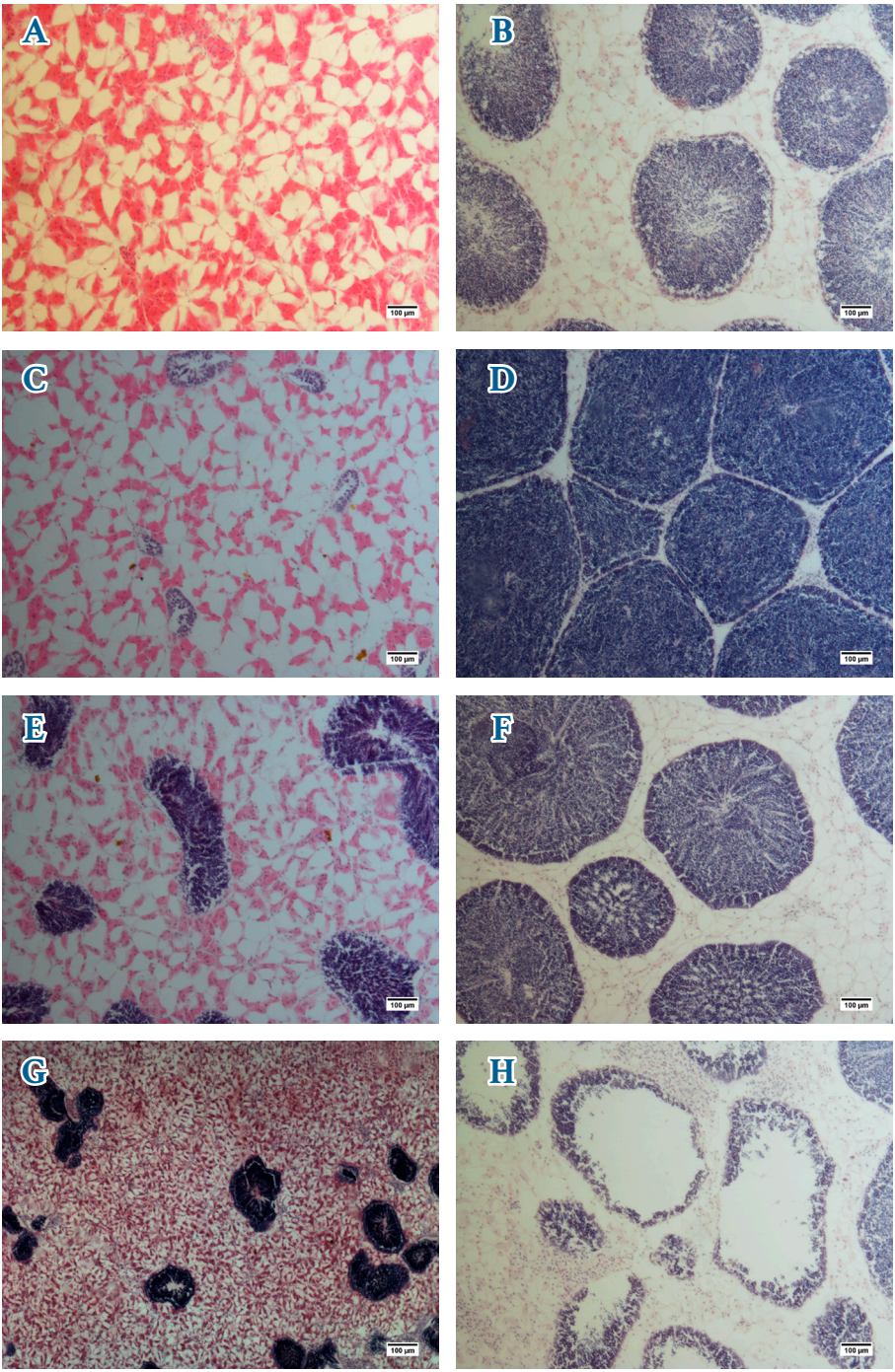
Figure 3 *Photomicrograph of male gonads from study sites. Gonad development stages in Mytilid and dreissenid, nomenclature adapted from (Seed (1975, 1976) by Hillman (1993)). 1. Resting/spent gonad (Stage 0 Inactive or undifferentiated) (**photomicrograph_A**); 2. Developing gonad: Stage 1 Gametogenesis has begun; no ripe gametes visible (**photomicrograph_ B**); Stage 2 Ripe gametes present; gonad developed to about one-third of its final size (**photomicrograph_ C**); Stage 3 Gonad increased in mass to about half the fully ripe condition; each follicle contains, in area, about equal proportions of ripe and developing gametes (**photomicrograph_D**); Stage 4 Gametogenesis still progressing, follicles contain mainly ripe gametes (**photomicrograph_ E**); 3. Ripe gonad: Stage 5 Gonad fully ripe, early stages of gametogenesis rare; follicles distended with ripe gametes; ova compacted into polygonal configurations; sperm with visible tails) (**photomicrograph_F**); 4. Spawning gonad: Stage 4 Active emission has begun; sperm density reduced; ova rounded off as pressure within follicles is reduced (**photomicrograph_G** and **photomicrograph_H**); Stage 3 Gonad about half empty (**photomicrograph_I**); Stage 2 Gonadal area reduced; follicles about one-third full of ripe gametes (**photomicrograph_J**); Spent gonad (Stage 0 Inactive or undifferentiated) (**photomicrograph_K**); Hemocitic infiltration (**photomicrograph_L**); Parasite (**photomicrograph_M**); Granulocitoma (**photomicrograph_N**)..*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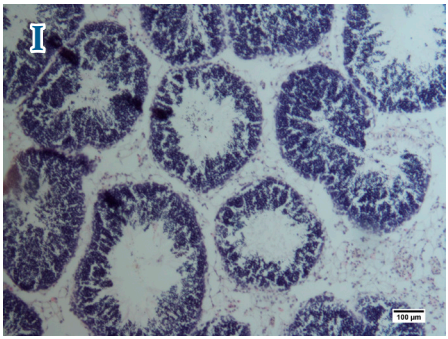
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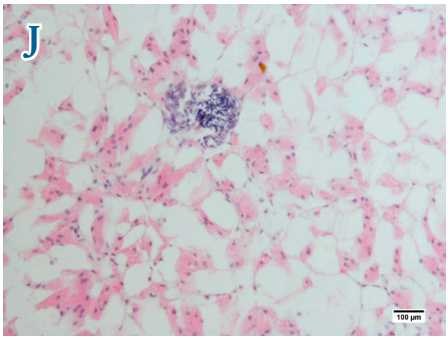
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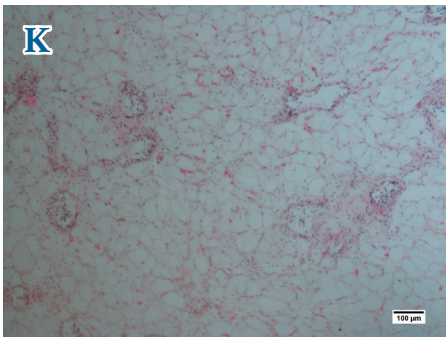
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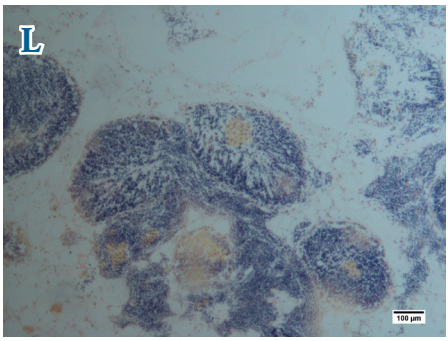
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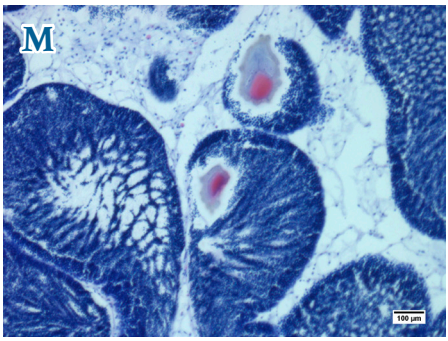
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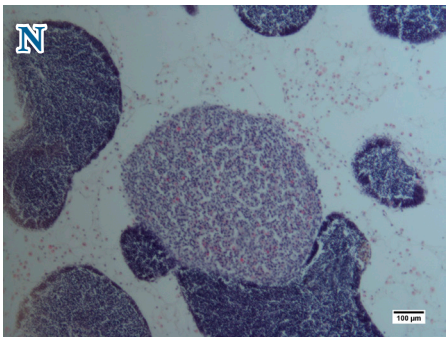
N
100 µm

Stereology

Gonad sections of 3-5 μm were examined with cellSens (Olympus) device with a microscope attached at a magnification of x240. Point counts (Weibel and Elias 1967) on adipogranular cells (ADG cells), vesicle connective tissue cells (VCT cells), developing and morphologically ripe gametes, areas of empty follicles resulting from spawning activity, as well as areas of pathologies were made on four independent fields of the same histological section, to quantify the volume fraction of the different tissue components (Pipe 1987). Stereological analysis was carried out using the techniques described by Lowe *et al.* (1982) and Pipe (1987).

An ImageJ (Rasband 1997-2009) routine was developed to overlay digital images of histological sections with a Weibel grid (Weibel *et al.* 1966). The Weibel grid contained 360 points and covered an area of 0.0034 cm^2. The distance between points (35 μm) was smaller than the average of the smallest cell type. The points touching cross sections of specific gonad component were enumerated. The mean area was calculated for each gonad component using modified Weibel formula (Haslob *et al.* 2013).

Statistics

Data were plotted using Sigmaplot (11.0) statistical and graphical software (Systat software) and statistical analysis was carried out using a statistical package (SPSS 17.0 for Windows®). When checking for significant differences in sex ratios, Gametogenic Index (GI) as well as partial volumes of gonad tissue cellular components only males and females were taken into consideration, undifferentiated and hermaphrodites individuals being excluded from the analysis. Data were tested for normality (Shapiro-Wilk *test*) and variance homogeneity (Leven's *test*). All comparisons were carried out using two-way ANOVA for homogenous variances and Tuckey's *test* as post hoc test, or Kruskal-Wallis and Mann-Whitney U *test* for heterogeneous variances. Wilcoxon Signed Ranks *test* was used to test differences between genders at each studied site. Sex ratio bias was studied using Chi-square Goodness-of-fit *test* (one variable) comparing total number of female and male mussels and normalizing for theoretical gender bias (1:1). Significant was established for all cases at $p < 0.05$.

Finally, principal component analysis (PCA) were run to reveal the association between environmental variables and to determine the main factors influencing variability.

RESULTS

Environmental parameters

No statistically significant differences were found in annual temperature cycles among sites (Two-way ANOVA; p=0.756), nor between years (Two-way ANOVA; p=0.064), starting in spring 2010 and ending in winter 2012. The variability in the water temperature along the Basque coast was mainly driven by the typical seasonal cycle (Two-way ANOVA; p<.000), and this seasonal cycle did not change between the two studied periods (Two-way ANOVA; p=0.504) (Figure 4A). During the winter surveys, temperature ranged from 10 to 15 ^{o}C (Tukey *test*; p<0.000). Maximum values were measured in summer (20-25^{o}C) (Tuckey *test*; p<0.000), and intermediate values in spring and fall (15-20^{o}C) (Tuckey *test*; p=0.994). Nerbioi estuary and its coastal zone presented the narrowest range of temperature variation, whereas Bidasoa estuary presented the widest (Figure 4A).

Regarding phytoplankton biomass (Figure 4B) along the Basque coast, statistically significant differences in the Chl-a concentrations were not detected, neither among seasons (Two-way ANOVA; p=0.111), nor between analyzed years (Two-way ANOVA; p=0.807). No interaction was observed between studied seasons at each analyzed year (Two-way ANOVA; p=0.517). Regarding spatial variability, no

significant annual differences were observed among sites, in either of the two studied periods (Kruskal-Wallis *test*; p=0.434 and p=0.451, respectively). However, Chl-a presented higher temporal variability in estuaries, compared to the open coastal sites (SD = 1.275 vs. SD = 0.5, respectively). At the open sites, Chl-a was fairly constant for most of the studied period, with values lower than 1 $\mu g\ l^{-1}$; Nerbioi coast reached the maximum Chl-a concentration in fall 2010 (1.1 $\mu g\ l^{-1}$) and Mutriku coast in winter 2011 (2.4 $\mu g\ l^{-1}$). In contrast, Chl-a peaked in estuaries during spring and/ or summer. The highest peaks were recorded in the Oiartzun estuary .

Salinity presented spatial variability in both studied periods (Kruskal-Wallis *test*; p=0.012 and p=0.012, respectively), whereas no significant seasonal effect in salinity was observed along the Basque coast in each analyzed period (Kruskal-Wallis *test*; p=0.996 and p=0.231, respectively) (Figure 4C). Salinity was close to 35 psu (practical salinity units) at both open coastal sites. It varied more in estuaries, especially in the Bidasoa estuary, which displayed significantly different salinity compared to the other studied sites (Mann-Whitney U *test*; $p<0.05$). The stations of Nerbioi and Oiartzun estuaries presented generally similar euhaline conditions (30-35 psu) (Mann-Whitney U *test*; p=0.200 and p=0.343 in each studied period, respectively). However, polyhaline

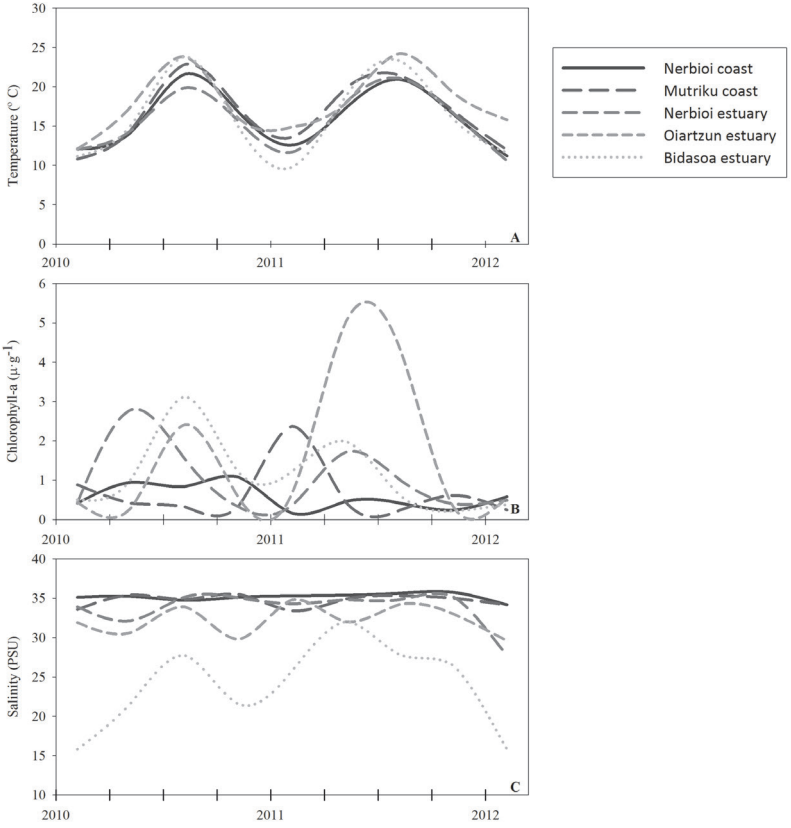

Figure 4 *Water temperature (A), Chlorophyll-a (B) and salinity (C) measured every three months during both of the studied periods*

conditions (18-30 psu) were usually found in the Bidasoa estuary, significantly different (Mann-Whitney U *test*; $p<0.05$) if compared to the other two estuarine sites.

Similar to salinity and Chl-a, nitrogen and phosphorous presented higher variability in estuaries compared to coastal zones (Figure 5A and 5B). Regarding TN cycle (Figure 5A), significant spatial differences were found in both studied periods (Kruskal-Wallis *test*; $p=0.016$ and $p=0.022$, respectively). Coastal sites presented significantly lower TN (Mann-Whitney U *test*; $p<0.05$). The highest TN concentrations were found in Oiartzun estuary, with values of approximately 150-160 μM (Figure 5A). In

Nerbioi and Bidasoa estuaries TN peaks were around 100 μM, whereas at coastal sites TN was always below 40 μM. Seasonal differences were not found for TN concentration in any of the studied periods (Kruskal-Wallis *test*; $p=0.313$ and $p=0.371$, respectively). Regarding TP cycle, significant differences were found among sites in both studied periods (Kruskal-Wallis *test*; $p=0.009$ and $p=0.007$, respectively). Coastal sites in open nearshore waters also presented significantly lower TP concentrations than estuarine sites (Mann-Whitney U *test*; $p<0.05$). Maximum concentration was found in Nerbioi estuary, with almost 5 μM (Figure 5B). In Oiartzun and Bidasoa estuaries TP peaks were

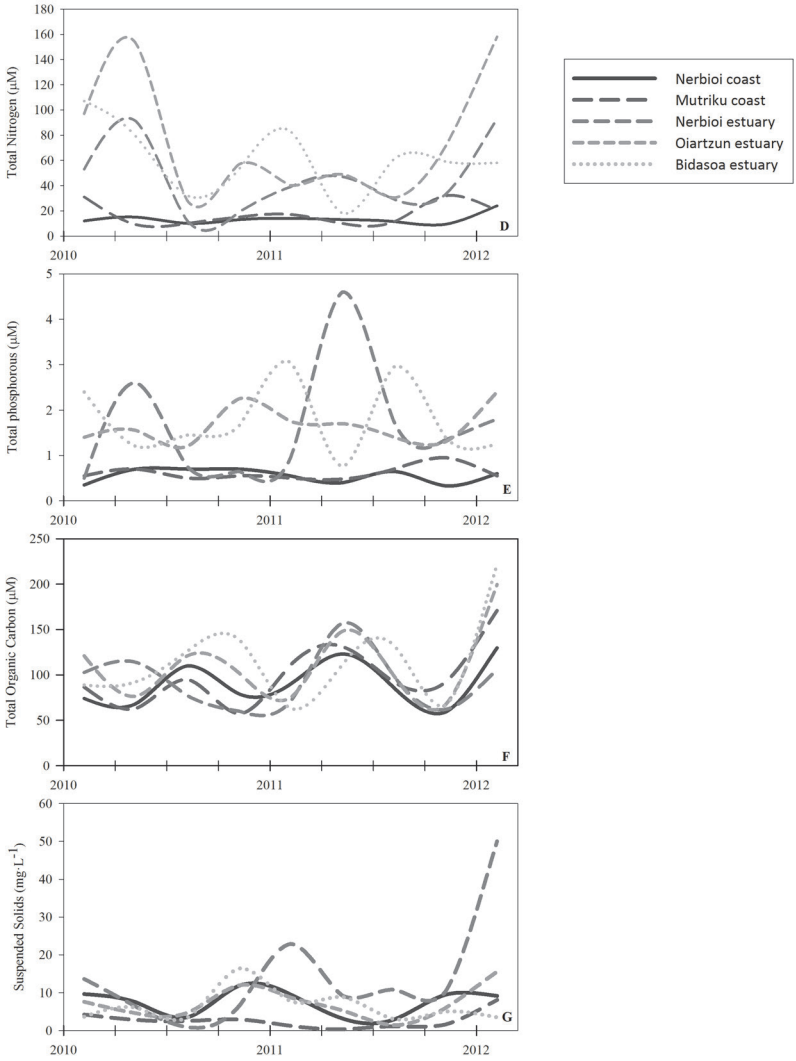

Figure 5 *Total nitrogen (D), total phosphorus (E), total organic carbon concentration (F) and suspended solids (G) measured every three months during both of the studied periods.*

between 2 and 3 μM. In coastal sites the highest TP concentration reached only 1 μM. No seasonal cycle in TP concentration was observed along the Basque coast, in either of the studied periods (Kruskal-Wallis *test*; p=0.852 and p=0.850, respectively).

TOC ranged from 50-150 μM. A spatial pattern was not detected (Two-way ANOVA; p=0.607), even though TOC was significantly different within sites each studied year (Two-Way ANOVA; p=0.028) (Figure 5C). Significantly higher TOC was observed during the second studied period (*t-test*; p=0.020). No significant interaction was observed between sites for each studied year (Two-way ANOVA; p=0.964). Despite the absence of a spatially significant pattern, estuaries presented higher concentrations and higher variability than coastal sites. Regarding seasonality of TOC along the Basque coast, TOC presented a seasonal pattern only in the second studied period (Kruskal-Wallis *test*; p=0.003), where similar high values were observed from spring (2011) to summer (2011) (Mann-Whitney U *test*; p= 0.056) and lower significant values (Mann-Whitney U *test*; p=0.016) were observed later in fall (2011). Thereafter, minimum significantly different TOC values were observed in winter (2012) (Mann-Whitney U *test*; p=0.032) compared to fall (2011). In fall, TOC values were similar to those observed in previous spring (2011) (Mann-Whitney U *test*; p=0.421).

SS along the Basque coast did not vary seasonally in either of the studied periods (Kruskal-Wallis *test*; p=0.060 and p=0.181, respectively). Values ranged from 0 to 50 mg l^{-1} (Figure 5D). Concentrations tended to increase in fall and in winter, and minimum concentrations were found in summer. Regarding sites, no spatial differences were detected in either of the studied periods (Kruskall-Walis *test*; p=0.121 and p=0.051, respectively). Nevertheless, the highest peaks were recorded in Nerbioi coast and the lowest values in Mutriku coast. Intermediate concentrations were found in the estuaries.

Spatial visualization of relationships among environmental variables

The results of PCA are shown in Figure 6. The analysis was performed with environmental data on Total Nitrogen (TN), Total Phosphorous (TP), Suspended Solids (SS), temperature, as well as salinity acquired during both studied periods. A large proportion of the variability (69%) was explained by two components. The first component was closely associated with TP (association coefficient: 0.864), TN (association coefficient: 0.847) and also with salinity (association coefficient: -0.586) and served to separate sampled coastal sites from sampled estuarine sites. The second component showed a close association among both temperature (association coefficient: 0.919) and suspended solids (SS)

(association coefficient: -0.750) and a looser association with salinity (association coefficient: 0.305) and served to separate sampled seasons.

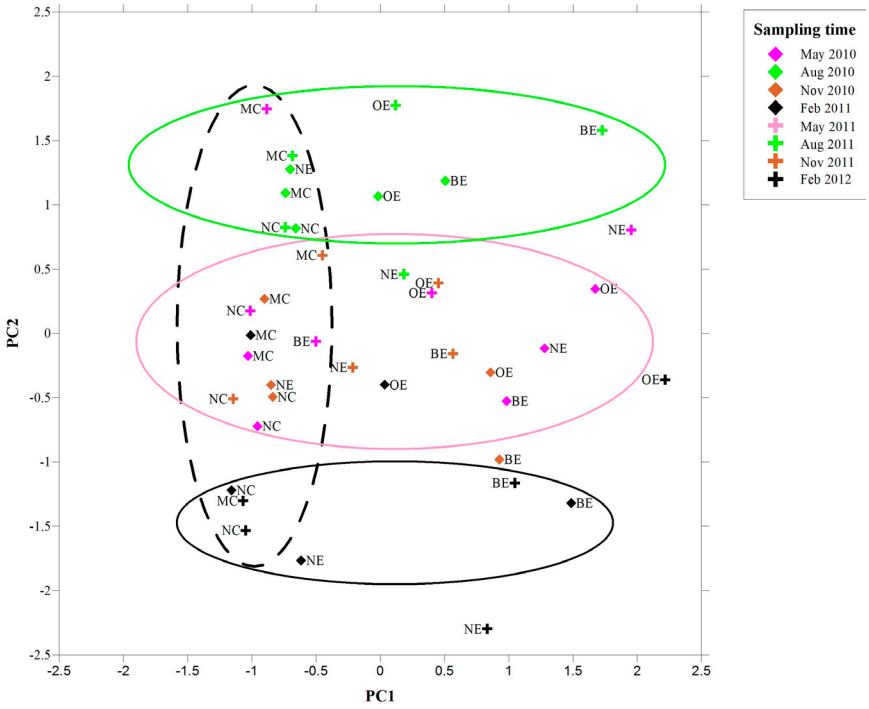

Figure 6 *Principal Component Analysis (PCA) of environmental data at each sampling time form the studied sites over two year periods.*

Sex-ratio

Sex-ratio values for each site and season are shown in Table 2. Statistically significant bias in the sex-ratio of the whole studied population (Chi-sq=0.793, p=0.373) was detected in comparison with the theoretical sex-ratio in mussel populations (1:1), with a female:male sex-ratio (0.94F:1M, n=919) for the total of both studied periods. Among sites, a statistically significant bias of more males was observed in Mutriku coast (Chi-sq=7.321, p=0.007). On the other hand, from the total of 1000 individuals observed and assessed for their gametogenic stage, only two hermaphrodite animals (0.2%) were found, both in Oiartzun estuary. These hermaphrodite individuals showed separate male and female follicles in their mantle tissue (See Figure 2M and 2N). Finally, an undifferentiated stage of development, where no gametes were present, was observed in 8% of analyzed individuals.

Table 2 Detailed description of sex ratio (female-to-male; F:M) data in studied mussel populations. Summary of Chi-square Goodness-of-fit Test performed to analyze the sex ratio of our studied populations in comparison to the theoretical sex ratio for mussels.

Studied System	*Female nº*	*Male nº*	*Sex Ratio*	*N*	*DF*	*Chi-Sq*	*p*
Nerbioi coast	98	100	0.98:1	198	1	0.020	0.887
Nerbioi estuary	90	89	0.99:1	179	1	0.006	0.940
Mutriku coast	75	112	0.67:1	187	1	7.321	0.007
Oiartzun estuary	89	84	1.06:1	173	1	0.145	0.704
Bidasoa estuary	94	88	1.07:1	182	1	0.198	0.657
Total	446	473	0.94:1	919	1	0.793	0.373

Gamete development and Gonad Index

Mussel populations along the Basque coast showed a marked seasonal cycle of gamete development in both studied years (Kruskal-Wallis *test*; $p<0.000$). Results are presented in terms of two reproductive years (period one: April 2010-Feb 2011; and period two: April 2011-Feb 2012).

Along the Basque coast (Figure 7), in the first studied period gametogenesis onset was observed from spring (2010) to *early* summer (2010). Subsequently, from *early* summer (2010) to *late* summer (2010) spawning stage was observed, with the end of the gametogenic cycle observed in *late* summer (2010). New gametogenesis was observed from fall (2010) to winter (2011). In the second period, gametogenesis proceeded in spring (2011) and spawning stage was observed from spring (2011) and continued until fall (2011), indicating a larger spawning period during this second summer. Consequently, in this second period the end of gametogenic cycle was observed later, in fall (2011). Gametogenesis onset was observed in winter (2012).

Microscopic observation of gonads from the two periods showed differences in gamete development patterns among sites (Kruskal-Wallis test; $p<0.000$). GI seasonal cycle clearly separated open coastal sites from western estuarine sites (Mann-Whitney U test; $p<0.05$), but

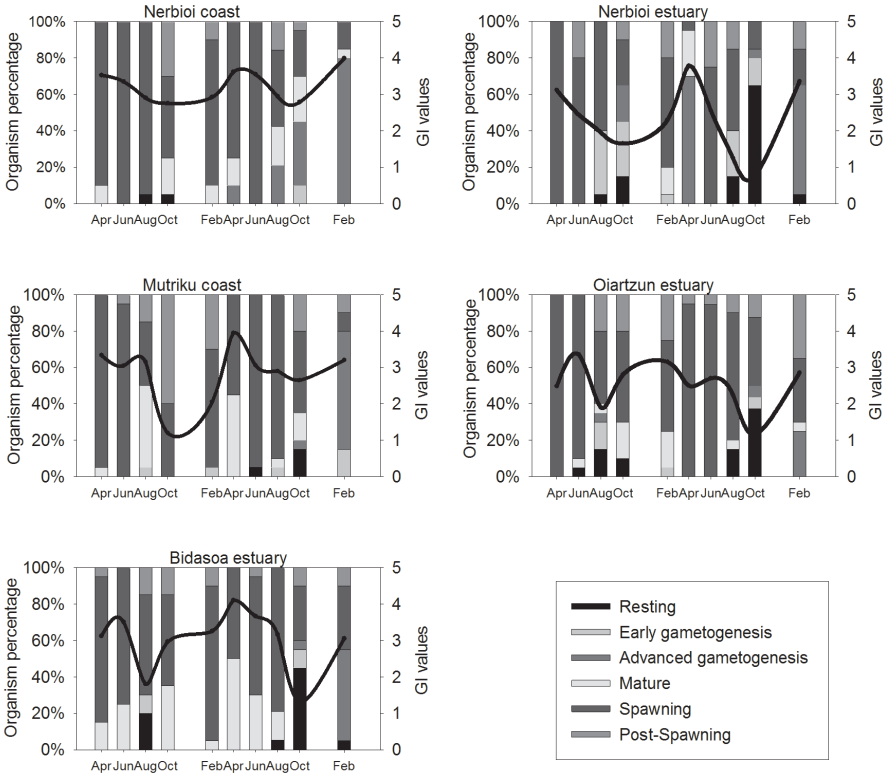

Figure 7 *The percentage distribution of gamete development phases (resting stage, early gametogenesis, advanced gametogenesis, maturity, spawning and post spawning stage) (stacked bars) and gonadic index (GI) values (black solid line) of mussel populations (Nerbioi coast, Nerbioi estuary, Mutriku coast, Oiartzun estuary and Bidasoa estuary) at each sampling time over two year periods.*

the seasonal cycle observed in Nerbioi coast and estuary did not differ significantly (Mann-Whitney U test; p=0.454). Regarding differences among estuaries, significant differences were also observed in the seasonal cycle between western estuarine site (Nerbioi estuary) and eastern estuarine sites (Oiartzun and Bidasoa estuary) (Mann-Whitney U test; p<0.000 and p<0.000, respectively).

At each analyzed site, in the two observation periods the seasonal cycles of gamete development and GI showed significant differences between genders (Wilcoxon Signed Ranks *test*; p<0.05). However, in Bidasoa estuary during the first studied period as well as in Nerbioi coast during the second studied period genders showed similar mean seasonal GI (Wilcoxon Signed Ranks *test*; p=0.080 and p=0.345, respectively).

Stereology

The relative proportion of mantle/gonad tissue cellular components are shown in Figure 8 and Figure 9, for female and male mussels, respectively.

The mantle/gonad tissue cellular component proportions (i.e., proportions of reproductive cells and reserve tissue: ADG and VCT cells) observed in the two studied periods along the Basque coast showed a marked seasonal cycle (Kruskal-Wallis *test*; p<0.000), whereas the proportion of haemocytes in gonads did not show any seasonality

in either of the two studied years (Kruskal-Wallis *test*; p=0.780 and p=0.885). The level of oocyte atresia showed a seasonal cycle in the first studied period (Kruskal-Wallis *test*; p=0.029) but not the following year (Kruskal-Wallis *test*; p=0.963).

With respect to reproductive cells along the Basque coast in the first studied period, highest proportions were observed in *early* summer (2010) and thereafter proportions decreased to minimum levels in *late* summer (2010), whereas in the second studied period highest proportion of reproductive cells were observed in spring (2011) and thereafter decreased to minimum levels in fall (2011).

Regarding reserve tissue, the proportion of ADG cells in the first studied period increased from *early* summer (2010) to *late* summer (2010). From *late* summer (2010) to winter (2011) the level of ADG decreased slightly but remained high. In the second studied period the proportion of ADG cells increased from minimum levels observed in spring (2011) to maximum levels observed in fall (2011). From fall (2011) to winter (2012) the level of ADG decreased but remained high. Regarding VCT cells, in the first studied period lowest proportions were observed in *early* summer (2010). Over summer the proportion of VCT cells increased to maximum levels, whereas from *late* summer (2010) to winter (2011), levels decreased slightly but remained high. In the

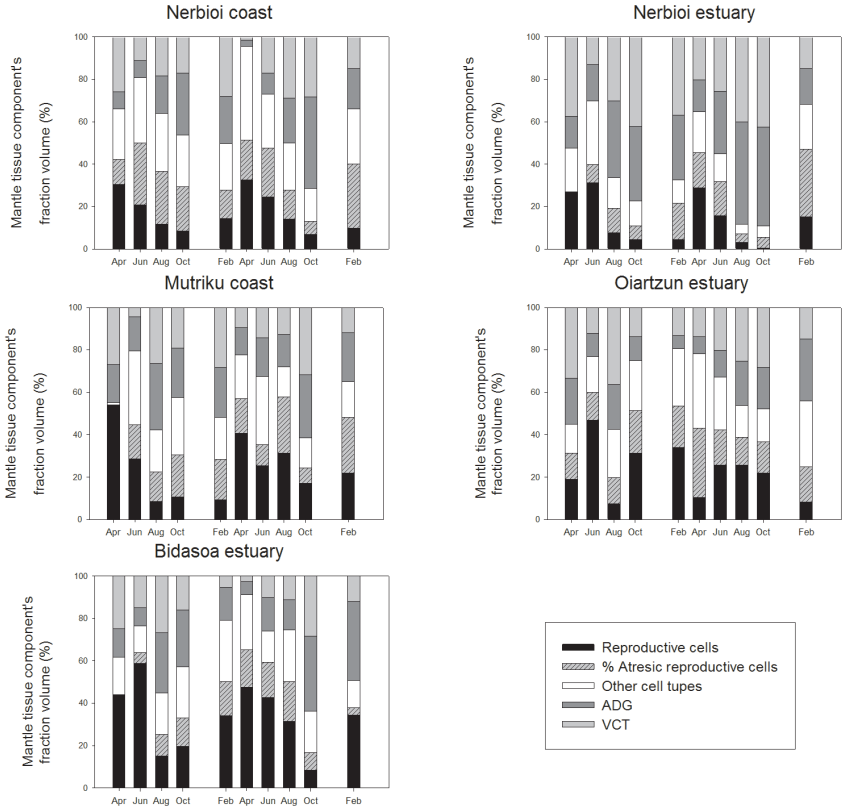

Figure 8 *Variation on female mussel mantle tissue components volume fraction (stacked bars) in the five studied sites. From top left to right (Nerbioi coast, Nerbioi estuary, Mutriku coast, Oiartzun estuary and Bidasoa estuary). Reproductive cells, atresic reproductive cell, other cell type, ADG (Adipogranular connective tissue) cells and VCT (vesicular connective tissue) cells partial volume at each sampling time over two year periods.*

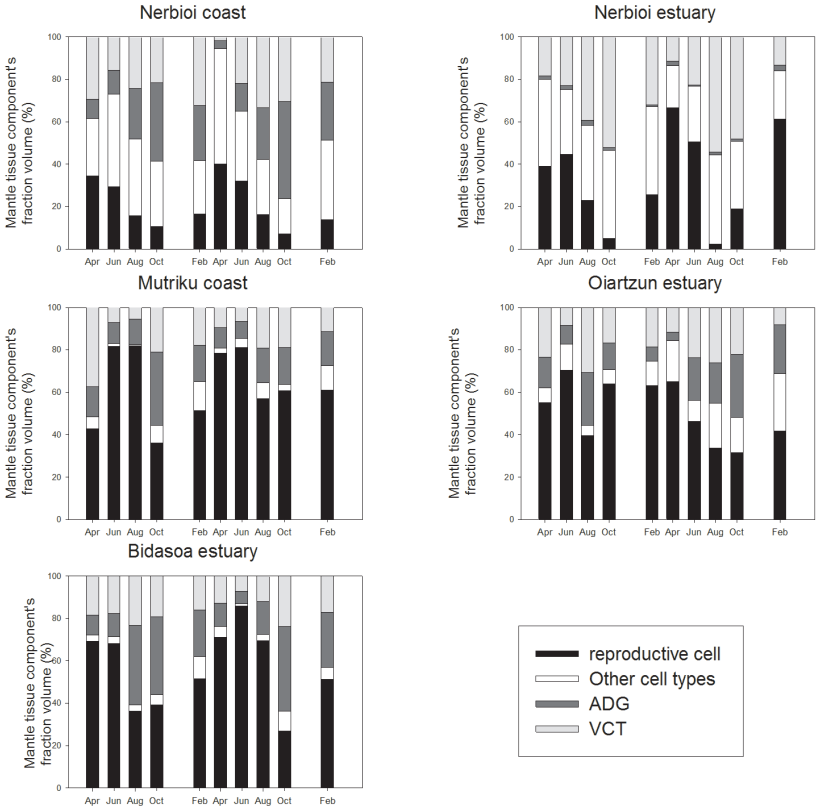

Figure 9 *Variation on male mussel mantle tissue components volume fraction (stacked bars) in the five studied sites. From top left to right (Nerbioi coast, Nerbioi estuary, Mutriku coast, Oiartzun estuary and Bidasoa estuary). Reproductive cells, other cell type, ADG (Adipogranular connective tissue) cells and VCT (vesicular connective tissue) cells partial volume at each sampling time over two year periods.*

following year, low proportions were observed in spring (2011). From spring (2011) to fall (2011) an increase in the proportion of VCT cells was observed, whereas from fall (2011) to winter (2012) the proportion of VCT cells decreased.

Concerning haemocytes, no seasonal cycle was observed but highest proportions of haemocytes were observed in fall in the two studied periods.

The seasonal cycle of gonad tissue cellular components (i.e., reproductive cells, reserve tissue cells and haemocytes) were significantly different among studied sites (Kruskal-Wallis *test*; $p<0.000$). Significant differences in the proportion of reproductive cells as well as in the proportion of ADG and VCT cells were observed between Oiartzun estuary and the other studied sites (Mann-Whitney U *test*; $p<0.000$). In addition, regarding reserve tissue cells, on one hand, significant difference in the proportion of ADG cells was observed between the two studied coastal sites, Nerbioi coast and Mutriku coast (Mann-Whitney U *test*; $p=0.011$). On the other hand, regarding VCT cells proportion, Bidasoa estuary, as observed with the other eastern estuary (Oiartzun estuary), showed significantly different VCT cells cycle compared to the other studied sites (Mann-Whitney U *test*; $p<0.05$).

Regarding haemocytes, significant differences were observed among

all studied sites (Mann-Whitney U *test*; $p<0.05$), with the exception of Nerbioi coast and estuary (Mann-Whitney U *test*; $p=0.737$).

Significant differences between genders at each studied site, over both studied periods, were observed (Wilcoxon Signed Ranks *test*; $p<0.05$). Nevertheless when each of the two studied periods was analyzed separately, Nerbioi estuary showed no difference between genders (Wilcoxon Signed Ranks *test*; $p=0.345$ and $p=0.080$). Regarding the seasonal cycle of reserve tissue cells (ADG and VCT cells), no significant differences were observed between genders at each studied site (Wilcoxon Signed Ranks *test*; $p>0.05$). To the contrary, regarding the seasonal cycle of haemocytes, significant differences between genders were observed in all studied sites (Wilcoxon Signed Ranks *test*; $p>0.05$).

Finally, the proportion of atresic oocytes observed in all sites and all samplings in each of the two studied periods was below 20%. Along the Basque coast neither a seasonal nor a spatial pattern in the proportion of oocyte atresia was observed (Kruskal-Wallis *test*; $p=0.394$ and $p=0.111$, respectively).

DISCUSSION

Influence of environmental conditions on the reproductive cycle

The timing and duration of the reproductive cycle of aquatic organisms, such as *Mytilus galloprovincialis* Lmk., in temperate regions, varies seasonally due to local environmental conditions, especially temperature and food availability (Seed 1976; Bayne 1976; Sastry 1979; Sunila 1981; Kautsky 1982; Lowe *et al.* 1982; Newell *et al.* 1982; Sprung 1983; Rodhouse *et al.* 1984; Villalba 1995; Múgica *et al.* 2015). On the Basque continental shelf, as in other temperate areas located at mid-latitude, the annual cycle of the Sea Surface Temperature (SST) shows a marked seasonality related strongly to atmospheric temperatures. There are two clearly defined seasons, winter and summer; and two transitional seasons, spring and fall. The SST differentiates the period of vertical mixing from the stratification period (Valencia *et al.* 2004; Fontán *et al.* 2008). This typical seasonal cycle in the SST was also found during this study.

Regarding food availability for successful reproduction, females and males require enough energy to carry out reproductive processes and indeed, the percentage of individuals at each gametogenic stage is related to food availability in the surrounding environment (Sastry 1979; Bayne and Newell 1983; Avellanal *et al.* 2002). When phytoplankton

availability decreases, mussels can use their own reserves (Bayne 1976) or alternative food sources, such as dissolved organic matter, bacteria and organic aggregates (Sastry 1979).

In the studied area, although statistical differences were not significant, Chl-a peaked in the estuaries during spring and summer, while lower peaks were found in fall and winter at the open coastal sites. Similar spatial patterns and seasonal cycles have been observed in previous studies of phytoplankton biomass dynamics along the Basque coast (Revilla *et al.* 2009; Garmendia *et al.* 2011). In the present study, the number of sampling campaigns conducted for Chl-a, which varies greatly at different temporal scales in coastal waters, probably was insufficient to achieve a higher statistical significance.

The highest peaks of Chl-a in the Basque estuaries are usually found in summer, when water residence time increases and insolation is higher (Orive *et al.* 2004; Garmendia *et al.* 2011). Similarly, regarding alternative energy sources (in special, organic carbon) on average the estuarine waters were richer than the exposed coastal sites. A consistent seasonal pattern was not found for total organic carbon. However, it was higher during winter and/ or fall, similarly than suspended solids concentration. This reflects the riverine origin of other energy sources than phytoplankton at the studied estuarine sites, as freshwater discharges in the southern

Bay of Biscay are higher during the cold seasons (Prego and Vergara 1998; Prego and Cobelo-García 2007).

Regarding salinity, both Mutriku and Nerbioi nearshore waters showed almost constant conditions, similar to the open sea, and thus, no metabolic stress would be expected there due to salinity. Salinity varied little in the Oiartzun and Nerbioi estuarine sites, in euhaline conditions. In the Bidasoa estuary, however, salinity was lower and more variable (polyhaline conditions). The salinity at Bidasoa estuary could influence the desired reproductive performance of wild mussel populations. In this sense, Jansen *et al.* (2007) indicated that, in the Bay of Biscay, temporary drops in salinity could induce metabolic depression in *Mytilus* species.

Principal Component Analysis results supported the experimental design (spatial and temporal sampling strategies). The near shore open coastal waters and the estuarine sites were clearly differentiated due to salinity and alternative food sources in water, whereas temperature cycle and suspended solid patterns served to describe the variability observed among sampled seasons.

Spatial and temporal variability in the reproductive cycle

Wide differences are found in the pattern of *Mytilus galloprovincilais* Lmk. gonad development of different geographical areas (Seed 1976; Da Ros *et al.* 1985). Indeed, the Gonad Index values obtained in this study

indicate that all stages can be found within the population at any season. However, differences between mussels located at nearby sites have also been reported, and are explained by environmental variables such as differences in food availability (Newell *et al.* 1982; Rodhouse *et al.* 1984; Ferrán 1992). The aforementioned environmental variables divided clearly summer and winter seasons and characterize those seasons as different in studied estuarine and coastal sites.

This study showed the same reproductive pattern as mussels from previous studies in the SE Bay of Biscay (Ortiz-Zarragoitia *et al.* 2011; Ortiz-Zarragoitia and Cajaraville 2010; Garmendia *et al.* 2010; Cuevas *et al.* 2015). Using stereological data from the present study, it can be concluded that animals undergo gametogenesis during winter months, deriving energy from stored reserves, as described by Pipe (1987).

In this study, levels of VCT cells were higher in spring and in fall. However, VCT cell levels were relatively high in all studied populations in both studied years. The eastern studied sites, Oiartzun and Bidasoa estuaries, showed high VCT cell levels from spring to fall. This may well have been related to the high food availability (Chlorophyll-a and TN) in that season in those estuaries. On both studied coastal sites, Nerbioi and Mutriku coasts, VCT cell levels rose from *late* summer to winter, in concordance with the high food availability (Chlorophyll-a

concentration and TOC) at these coastal sites. By contrast, the western studied estuary, Nerbioi estuary, showed higher levels of VCT cells from *late* summer to winter in the first studied period, while in the following year, high levels of VCT cells were observed from *late* summer (2011) to fall (2011). Nevertheless, non-phytoplanktonic food sources (TOC level or other sources such as SS) seem to be related to this seasonal reserve tissue production at Nerbioi estuary, as in the present study, estuaries showed higher food sources (Chl-a) during spring and summer seasons. No differences in reserve tissue, both ADG and VCT cells, cycles were detected between genders at each sampled site.

In this study most studied populations showed one spawning period in 2010 and another one in 2011, both of which typically started in spring and ended in *late* summer. Over the two-year period, however, in certain sites, the spawning period ended in *early* summer. For instance, in both the Mutriku coast and Oiartzun estuary in the first studied period (2010), and in both the Nerbioi coast and estuary in the following year (2011), the spawning period was reduced from spring to *early* summer or just to *early* summer. In Mutriku coast and Oiartzun estuary the reduced spawning period is likely to be caused by low food availability observed in both coastal and estuarine sites in *late* summer (2010) in the first studied period, compared to the following *late* summer (2011). In

both Nerbioi coast and estuary, high riverine input from Nerbioi river maintains a high concentration of Chorophyll-a in summer. The input in 2010 may not have been enough for the active reproductive period to continue until the end of summer.

In contrast to the reduced spawning period described above, in other sites two reproductive cycles were observed. For instance, in the Bidasoa estuary in the first studied period (2010), two spawning periods were observed (from spring to *late* summer, and another in the fall), while in the following year (2011), only one was observed. This year-to-year variability among abiotic factors create variability in the gonad cycle of individuals of the same site (Seed 1976; Ferrán 1992) and as a result fall spawning may occur some years (Andreu 1958; Aguirre 1979; Villalba 1995). A second spawning peak has been described previously in the fall in mussels from the Basque coast (Garmendia *et al.* 2010; Múgica *et al.* 2015). The second spawning period observed in Bidasoa estuary during the fall of 2010, may also be related to the high presence of spawning individuals analyzed throughout both studied periods. Furthermore, this continuous spawning of individuals may be induced in summer by temperatures above thermal stress (Múgica *et al.* 2015), and/ or by temporal drops in salinity occurring at any time throughout the year.

No significant female:male sex-ratio bias was measured on Nerbioi

coast, nor in Nerbioi, Oiartzun and Bidasoa estuaries, and the resulting ratio was close to that of 1:1, consistent with findings of Ortiz-Zarragoitia *et al.* (2011) in the SE Bay of Biscay. By contrast, significant sex-ratio bias in favor of males was measured on Mutriku coast. In some natural bivalve populations there is a higher percentage of females than males (Mackie 1984; Toro *et al.* 2002). The degree of bias is a characteristic property of the female parent, as mating of the same female with different males produces the same sex-ratio, but mating of the same male with different females produces different sex ratios (Kenchington *et al.* 2002).

Both sexes were found to display relative similarities in the timing of reproductive cycles. By contrast, on the Mutriku coast and in the Oiartzun estuary there was not such a synchrony between sexes. On the Mutriku coast, in addition to the aforementioned sex-ratio bias in favor of males, male mussels also showed differences to female mussels in the timing of reproductive cycles, and hence, spawning. Despite these differences in the timing of spawning, relative common spawning periods between both genders were observed on the Mutriku coast (i.e. *early* summer in the first studied period, and from spring to *late* summer in the following year). In the Oiartzun estuary, synchronized spawning periods between both genders were also observed (i.e., from spring to *early* summer in the first studied period, and in spring in the

following year). Furthermore, it was at this particular site that the only two hermaphroditic individuals of the study were found. Despite these apparent limitations, the populations' physiology was healthy and in Oiartzun estuary environmental conditions were observed to be favorable in the fall and winter in the first studied period (2010). In addition, the low hermaphroditic level found at this site is in concordance with previous data reported by Sunila (1981) for *M. edulis*; by Lubet (1959) for *M edulis* and *M. galloprovincialis* in different geographical areas; and by Ortiz Zarragoitia *et al.* (2011) and by Cuevas *et al.* (2015), in the SE Bay of Biscay for *M. galloprovincialis* Lmk. specie.

When mature female gametes are not effectively spawned, these gametes are broken down at gonoduct level without being released (Suarez-Alonso *et al.* 2010). This occurrence, oocyte atresia, is caused by unfavorable conditions such as low temperatures or low food availability, which ultimately prevent mature female mussels from spawning (Sastry 1979; Suarez-Alonso *et al.* 2010). Similarly, Suarez-Alonso *et al.* (2010) found that in *late* summers, with high temperatures and less proportion of gonads occupied by gametes, a rise in atresic oocytes in mussels was produced, indicating the end of the gametogenic cycle. An increase in the normal levels of oocyte atresia have also been attributed to pollutants (Puy Azurmendi *et al.* 2010) or thermal stress (Múgica *et al.* 2015) of

mussels inhabiting the Basque coast. Indeed, Ortiz-Zarragoitia *et al.* (2011) suggested that pollution-causal production of atresic oocytes increased in association with higher defense metabolism and further energy consumption. Hence, the trend on increase in oocyte atresia in observed in this study, is most likely due to the end of gametogenic cycle. The atresia level was higher from *late* summer until winter, corresponding with the end of the spawning period and resting period, respectively. Whereas, minimum levels were found from spring to *early* summer, during active gametogenic period and spawning time. Moreover, the level of oocyte atresia in the studied populations along the Basque coast was normal and therefore oocyte atresia caused by pollution could not be confirmed in this study.

Conclusions

The present study provides a detailed description of five mussel populations inhabiting the coast of the SE Bay of Biscay (two in open coastal waters and three in estuarine sheltered waters). The reproductive cycle of the mussel populations inhabiting those sites was assessed using Gonad Index and stereology. Along the Basque coast gametogenesis onset was observed from winter to spring, with spawning occurring from spring till summer. This was followed by a resting phase and an increase in reserve tissues (ADG cell) in fall. Generally, mature mussels

stayed ripe during winter until favorable environmental conditions were met in spring. Therefore, it can be suggested that in order to secure and protect potential maximum natural reproductive outputs, mussel harvesting should be avoided from spring to the end of summer, on the Basque coast. It was observed in the two-year period of the study that the reproductive cycles of the studied populations did not follow a consistent pattern. Indeed, differences were found among sites, and year-to-year variability was observed at each site. In addition, at two of the studied sites asynchrony was observed between genders in the timing of spawning. Nevertheless, these timing differences did not prevent mussels at those sites from reproducing successfully. Moreover, mussels inhabiting these studied coastal and estuarine sites are considered to display normal reproductive cycles. Hence, these sites have the potential to become main sources of wild mussel spat for future expected commercial mussel farming in the Basque Country. Furthermore, among the studied sites, the high food availability found in the Bidasoa estuary is propitious for achieving long and healthy active reproductive periods in mussels.

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CHAPTER 3

SETTLEMENT AND RECRUITMENT PATTERN VARIABILITY OF THE MUSSEL (*Mytilus galloprovincialis* LMK.) FROM SE BAY OF BISCAY (BASQUE COUNTRY).

SUMMARY

The annual settlement and recruitment patterns of *Mytilus galloprovincialis* Lmk. were assessed using collector ropes deployed at five sites along the Basque coast inhabited by wild mussel populations. The study was carried out over two periods of ten months each in three estuarine and two coastal sites, and enabled the discovery of a potential relationship between observed primary settlement and the spawning period of wild mussel populations, sheding light on the recruitment of these populations. Primary settlers were present throughout the year with highest abundances from early summer to fall. A spatial settlement pattern was also observed, with eastern estuaries showing higher settlement rates than western ones and coastal sites showing intermediate settlement rates. These spatial and temporal settlement variations could be partially explained by seasonal fluctuations in environmental factors, such as temperature and nutrients, as well as by spawning differences observed at these estuarine and coastal sites. Some inter-annual variability of settlement and recruitment was observed, which was consistent with the inter-annual variability of the environmental factors. The recruitment of juvenile mussels was shown to be more determined by post-settlement processes than by initial settlement abundance. Indeed, there was a substantial loss of post-larvae during fall

and winter, which was due to these post-settlement processes. Thus, it is advised that ropes be collected earlier to avoid such losses. Little space is available (i.e., due to user conflict) on the Basque Coast. Despite these limitations, these sites, particularly Bidasoa estuary, could be suitable for future seed gathering for the mussel aquaculture industry in the Basque Country.

Key words: *Mytilus galloprovincialis* Lmk., artificial collector ropes, primary settlement, recruitment, larvae, Bay of Biscay

INTRODUCTION

The development of shellfish farming industries, such as mussel farming, requires a reliable supply of sufficient seed (Mason 1976) and thus these industries are often located in areas where spat are readily available. An experimental suspension mussel longline culture system (Langan 2000) was planned by the Basque Government to test the feasibility of culturing shellfish in the open waters of the SE Bay of Biscay (Basque Country). Small mussel populations can be found along the Basque coast; yet, the spatial and temporal dynamics of mussel spat arriving to this coast are highly variable and poorly understood.

The life cycle of *Mytilus galloprovincialis* Lmk. involves a pelagic larval stage, named veliger stage (>225μm), in which larvae show simple morphology, with a ciliated swimming organ, a functional gut and a shell. These provide the larvae with the basic requirements for a pelagic phase with a high dispersal capability, which in some cases can last over a month (Cáceres-Martínez and Figueras 1998a). Planktonic veliger larvae have a limited swimming capacity (< 0.1 $cm \cdot s^{-1}$ (Young 1995)). However, larvae can regulate along-shore and cross-shore displacements with vertical migrations between water layers flowing in opposite directions (Poulin *et al.* 2002; Queiroga and Blanton 2004; Marta-Almeida *et al.* 2006). Local oceanographic conditions and weather patterns contribute

to the dispersion and concentration of larvae at various spatial and temporal scales (Kingsford 1990; Martel *et al.* 1994). As a result, larvae can be dispersed over seas of 10s to 100s of km away from parental mussel beds (McQuaid and Phillips 2000; Becker *et al.* 2007). Nevertheless, those vertical migrations may allow the maintenance of larvae close to parental habitats (Sponaugle *et al.* 2002), even along the open coast (Shanks and Shearman 2009, Morgan and Fisher 2010).

Veliger larvae (>225μm) metamorphosis leads to pediveliger larvae (>470μm), which possess a large velum used for swimming and feeding, a foot used for crawling and a byssus system, capable of secreting simple byssal threads (Bayne 1976). The individual at this pediveliger larvae stage must select a suitable substrate for future sessile existence (Bayne 1971). Bayne (1964) termed "settlement" as the entire process involving crawling behavior, secretion of byssal threads as well as the attachment of larvae to a substrate. Thus, this settlement process links pelagic and benthic stages (Menge 1992) as it involves the initial attachment of larvae to the substratum and can significantly influence the final distribution and abundance of adult mussel populations (Bownes and McQuaid 2009).

In order to choose the most suitable substratum for survival, marine invertebrate larvae settle in response to a range of chemical and

physical factors (Hunt and Scheibling 1996). Metamorphosis can even be delayed for months until a suitable substratum is found (Lane *et al.* 1985). Mussel larvae also settle in response to natural cues such as biofilms, macroalgae, and conspecifics that can induce settlement (Gribben *et al.* 2011). For instance, it has been described that *Mytilus edulis* L. (Bayne 1976) larvae initially settle onto filamentous substrates such as algae and hydroids. This initial attachment is called primary settlement (i.e., primary settlers; <0.5mm). Later, larvae can detach and re-attach. This is called secondary settlement (i.e., secondary settlers; >0.5mm) (Bayne, 1976) and can occur in a different zone or substrata (Alfaro 2006b) and move to establish themselves on existing mussel beds (Bayne 1964). This attachment and re-attachment may occur several times before juvenile mussels enter maturity (Bayne 1964).

The variability of mussel settlement relies on the timing and magnitude of larval supplies in addition to the physical conditions found by the animal in its environment. Initial larval supply depends on spawning periods and plankton blooms. Settlement, however, depends on various physical environmental conditions during both the pelagic and benthic stages. Factors influencing settlement in the pelagic stage are associated with shoreline topography (Roughgarden *et al.* 1988; Bertness *et al.* 1996; Archambault and Bourget 1999) and include ocean currents,

wind patterns and water temperature (King *et al.* 1990; Kingsford 1990; Petraitis 1991; Hunt and Scheibling 1997, 1998). Once closer to the shore, factors influencing settlement on the benthic stage are associated with tidal movements, wave action, substratum availability and tidal height (Gaines and Roughgarden 1985; Bertness *et al.* 1992; Vargas *et al.* 2004).

Several comparative studies have found that temperature, salinity and food availability are major exogenous factors that affect settlement and development of *mytilids* (Riisgard *et al.* 1980; Jespersen and Olsen 1982; Manahan *et al.* 1983; Sprung 1984). Indeed, settlement differences have been found when comparing habitats inside and outside bays and estuaries (Kaustky 1982; Petraitis 1991; Fuentes and Molares 1994; Snodden and Roberts 1997). Such differences can be related to differences among these exogenous factors. In order to analyze the settlement pattern along the Basque coast, the present study evaluated some sites with oceanic influence and some estuarine dominated sites.

Mussel seeds for culture can be collected either from natural mussel beds inhabiting the shore or alternatively, with artificial collectors, such as hanging ropes. Babarro *et al.* (2000) found that mussel seeds from artificial collectors have higher growth rates than naturally collected mussel seeds from the shore, when both were subsequently transferred to a culture system. These authors explained that mussel seeds from

artificial collectors are well adapted to permanent submersion which enables them to grow faster.

The selection of a preferred surface by the larvae can be related to characteristics of the artificial substrate, such as the surface roughness and microtexture (Berntsson *et al.* 2000; Köhler *et al.* 1999; Scardino and de Nys 2004; Schumacher *et al.* 2007a, b) and the number of adhesion points on the surface of the substrata to which the larva can adhere more strongly (Callow *et al.* 2002; Scardino *et al.* 2008). Indeed, the physical complexity of artificial substrates enhances the settlement of mussels (Filgueira *et al.* 2007; Walter and Liebezeit 2003). Nevertheless, this complexity is limited, as the larvae prefer surfaces with structures slightly smaller than the dimensions of the propagules of the larvae. Thus, if the structure of the surface for settling is slightly smaller than the dimensions of settling propagules, this substratum will be avoided (Aldred *et al.* 2010; Berntsson *et al.* 2000; Callow *et al.* 2002; Scardino *et al.* 2008). Further, the artificial surface topography of the substratum also affects wettability of the own rope, which in turn enhances larval settlement (Gribben *et al.* 2011).

The term "recruitment" refers to the number of larvae that have settled and survived for a certain period of time including a period of post-settlement mortality (Keough and Downes, 1982). The veliger

to pediveliger larvae metamorphosis occurring during the settlement, which results in a high rate of post-settlement mortality in mussel communities (Gosselin and Qian, 1997; García- Esquivel *et al.,* 2001). This post-settlement mortality may be the cause of large-scale differences in the composition and structure of the mussel communities inhabiting the shore, and may obscure previous settlement patterns (Osman and Whitlatch 2004). This recruitment to the shore is one of the key factors influencing adult populations with a planktonic larvae phase. Thus, in order to understand the pre-settlement process and the potential connectivity pathways between mussel populations, the temporal and spatial study of settlement patterns are widely accepted as indirect methods (Wing *et al.* 1995; Peteiro *et al.* 2011).

The aim of the present study was to clarify the time and duration of the mussel settlement season and the identification of the best sites for setting out collectors along the Basque coast. The importance of these findings is discussed both in terms of the potential use of suspended ropes at these sites to predict spat-falls and of the possibility of collecting newly settled mussel spats for being transported directly to future mussel culture systems, offshore. Further, this information along with recent insights into the adult reproductive cycle (chapter 2) improves our understanding of the mussel population dynamics and will assist

the development of strategies for ecosystem-based aquaculture industry management (Leslie and McLeod, 2007).

MATERIALS AND METHODS

Study area and location of mussel populations

This study was conducted at two coastal (Nerbioi and Mutriku coasts) and three estuarine (Nerbioi, Oiartzun, and Bidasoa estuaries) locations (Figure 1). These sites have been selected to contain large wild mussel populations and represent different water systems along the Basque coast (see chapter 2).

Sampling Strategy for mussel settlement

Experimental collector ropes were deployed near mussel beds in these five sites along the Basque coast (Figure 1 and Table 1). These structures for spat collection were each composed of a top and a bottom frame. The top frame was horizontally suspended 1m below the surface and attached to two buoys. Attached to this metal frame were three vertically suspended collector ropes of 2m length. These collector ropes employed for mussel seed collection in industrial cultivation were tested. The distance between ropes was approximately 50 cm. Parallel to, and

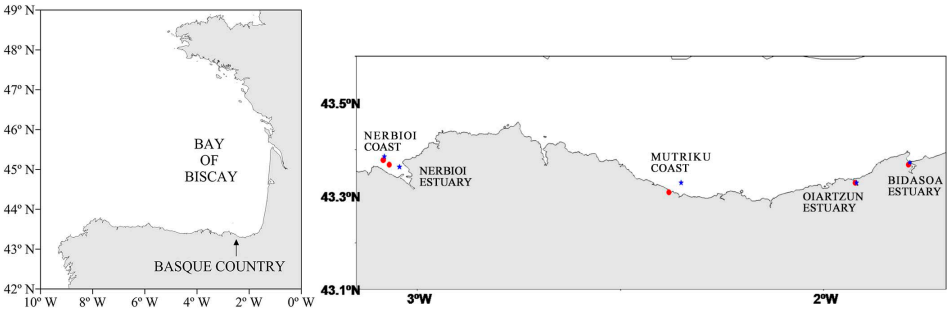


Figure 1 *Geographical location of the sampling stations used for the deployment of mussel collector ropes (red spots) and environmental variables analysis (blue crosses): Nerbioi coast, Nerbioi estuary, Mutriku coast, Oiartzun estuary and Bidasoa estuary .*

Table 1 The geographical location of the sampling sites used for mussel populations .

Studied system	***Mussel population***	
	Latitude	***Longitude***
Nerbioi coast	43.3775	-3.084
Nerbioi estuary	43.3535	-3.0792
Mutriku coast	43.3078	-2.3808
Oiartzun estuary	43.328	-1.9229
Bidasoa estuary	43.3679	-1.7915

in contact with each rope, four pieces of 25cm long individual rope were individually attached with cable tiers. The ends of the ropes were then attached to a similar horizontal metal frame. This bottom frame was moored to the seabed using a chain with a weight at its base.

The study was done over two separate periods of 10 months each, the first from April 2010 to February 2011 and the second from April 2011 to February 2012. As settlement is influenced by seasons, samples were taken in each season. Moreover, as summers are of key importance in the reproductive cycle and spat collection in temperate regions, two samples were taken, one at the beginning (June) and one at the end of summer (August) in both studied years. Thus, samples were collected in April, June, August, October and February, respectively.

At each sampling period and site with the help of scuba divers, one of the four pieces of 25cm rope was taken from each of the three collector ropes. At the end of each studied period the 2m long remaining ropes were collected and 25cm of each were taken for analyses in the laboratory. Samples were kept cold in liquid nitrogen until arrival at the laboratory, where they were thawed. Ropes were then frayed to remove all individuals from a 5cm-section of each rope. Since the mussels were attached to the rope fibers, they were detached by scraping, with the help

of a needle and tweezers and a wash bottle. Subsequent cleanings were conserved in a 5l beaker. Water containing the sample was filtered using a 50μm pore-side length mesh. The bivalve larvae were then separated from the rest of the planktonic organisms under an inverted microscope and larvae were collected with a crystal pipette and preserved in 70% ethanol.

Mytilid larvae were photographed and shell lengths of these larvae were measured to the nearest 0.1μm using cellSens (Olympus) device with a microscope attached to it. Mussel seed shell lengths along the anterior–posterior axis to the nearest 0.1mm were determined using a caliper.

Larvae identification

Identification chart

Previously photographed and posterior genetically tested (Inue *et al.* 1995) *Mytilus galloprovincialis* Lmk. larvae from the settlement study of different sizes (veliger stage (>225μm), pediveliger (>470μm), and post-larvae stage (>1mm)) were made to obtain an easy following *Mytilus galloprovincialis* Lmk. identification chart.

The identification of veliger larvae (0.225-0.470mm) was possible to genus (*Mytilus*) using keys and observations from Werner (1939),

Rees (1950), Lutz and Hidu (1979) and Le Pennec (1978 and 1980), as well as the present *Mytilus galloprovincialis* Lmk. identification chart. Marigomez *et al.* (2007) and Garmendia *et al.* (2011) described mussels inhabiting the south Bay of Biscay as *Mytilus galloprovincialis* Lmk. Thus, all *Mytilus* pediveliger larvae stage (0.470-1mm) were considered *Mytilus galloprovincialis* Lmk. Finally, post-larvae (> 1mm) were easily identified as *Mytilus galloprovincialis* Lmk.

DNA extraction and PCR

Well conserved and unopened mussel larvae were selected for DNA based species identification and preserved separately in ethanol and frozen at -80ºC until DNA extraction. DNA from pieces of mussel tissue (i.e., plantigrade size mussel) or from whole mussel larvae (i.e., veliger and pediveliger size mussel) was purified and extracted following the protocol Wizard® Genomic DNA Purification Kit (Promega) with the following modifications: 20 µgr of sample was incubated at 55°C for 3 hours in 600 µl lysis buffer. The buffer contained 600µl Nuclei Lysis solution, 0.5 mM EDTA, 0.5% SDS and 20 $\mu g \cdot ml^{-1}$ Proteinase K. Extracted DNA was re-suspended in 50 µl of sterile Milli Q water and stored at - 20°C. DNA concentration was checked using the NanoDrop (ND-1000 spectrophotometer)®, and the purity of DNA was assessed

through the ratio of absorbance at 260 and 280 nm. A ratio of ~1.8 was accepted as "pure" for DNA. Amplification of the non-repetitive domain of the Glu50 gene was done as in (Inoue *et al.* 1995). The PCR was carried out by adding 10 ng of DNA to the 20 µl PCR reaction mixture using Taq polymerase (KAPA HOTStart) in a Thermalcycler C1000 dual (BIORAD). Primers used for the amplification were Me15- 5'-CCA GTA TAC AAA CCT GTG AAG A-3'-and Me16- 5'-TGT TGT CTT AAT AGG TTT GTA AGA-3'- and PCR conditions were the following: a denaturation step at 95°C for 3 min, amplification during 35 cycles at 95°C for 15s, 56 °C for 30s and 72 °C for 3s and a final elongation step at 72 °C for 10 min. Amplified products were checked in 1.5 % agarose gel. 2 µl of PCR product was mixed with the loading dye solution containing bromophenol blue (BPB) and xylene cyan 01 and viewed using a computer-aided software program, ImageLab, provided with the gel analyzer ChemiDoc XRS (BIORAD). Electrophoresis was continued until BPB reached the end of the gel.

Statistics

Depending on measured size, mussels were divided into primary and secondary general classes. Primary settlers are those individuals with a shell length of <0.5mm, while secondary settlers are those individuals with a shell length of >0.5mm.

Data were plotted using Sigmaplot (11.0) statistical and graphical software (Systat software) and statistical analysis was carried out using a statistical package (SPSS 17.0 for Windows®). Data on numbers of primary settlers as well as the total accumulated number of mussel larvae (primary plus secondary settlers) settled on collector ropes were log-transformed to meet normality (Shapiro-Wilk *test*) and homoscedasticity (Leven's *test*) assumptions. Two-way analysis of variance (ANOVA) was applied to test differences in settlement among seasons as well as the differences between studied years along the Basque coast. In addition, two-way analysis of variance (ANOVA) was applied to test differences in annual settlement among studied sites as well as these annual differences between years. Both were followed by pair-wise multiple comparison procedures (Tukey's *test*). To account for multiple testing, Bonferroni correction was used. The *late* summer samples of Bidasoa estuary in the first studied period as well as the *early* summer samples of the Oiartzun estuary in the second studied period were lost in the laboratory and thus, these data are missing from the study. As a result, no equal size samples were compared on both of the studied periods and sum of square type IV was used (Zar, 1984). Kruskal-Wallis *test* was carried out for heterogeneous variances.

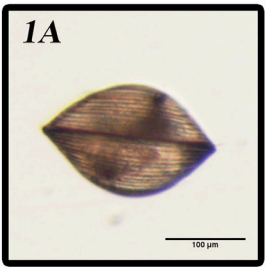
RESULTS

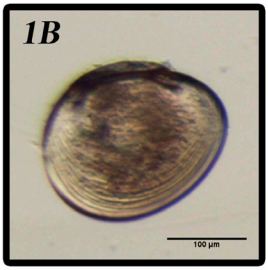
Larvae identification

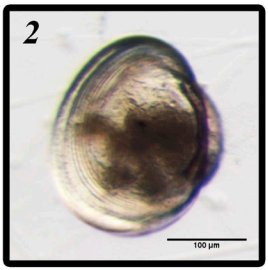
Identification chart

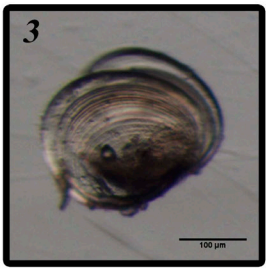
Previously photographed and posterior genetically tested (Inoue *et al.* 1995) *Mytilus galloprovincialis* Lmk. larvae from the settlement study of different sizes (from veliger stage (>225µm) to post-larvae stage (>1mm)) used in the identification chart are shown in Figure 2.

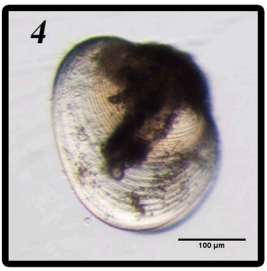
Figure 2 *Mytilus galloprovincialis Lmk identification chart. Photomicrographs of genetically tested (Inoue et al. 1995) Mytilus galloprovincialis* Lmk. *larvae from the settlement study of different sizes (from veliger stage (>225µm) to post-larvae stage (>1mm)).*











VELIGER:

1. Oiartzun estuary early summer 243.53µm

2. Oiartzun estuary early summer 252.19µm

3. Oiartzun estuary early summer 252.19µm

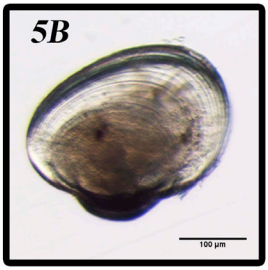
PEDIVELIGER:

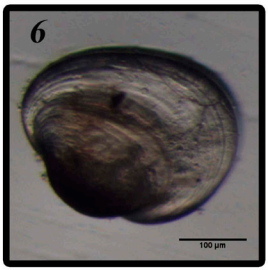
4. Mutriku coast early summer 300.59µm

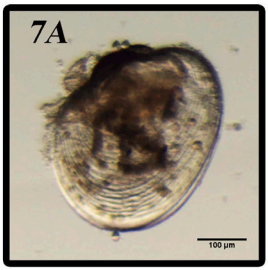
5. Mutriku coast early summer 313.66µm

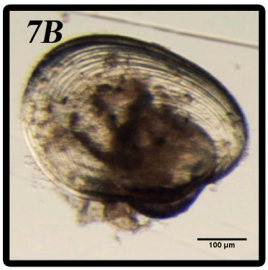
6. Oiartzun estuary early summer 314.39µm

7. Nerbioi estuary





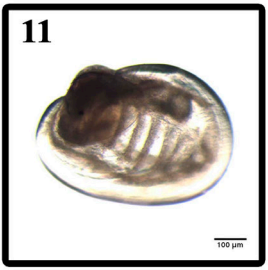




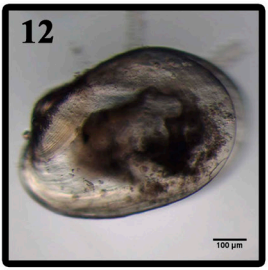
8. Oiartzun estuary
early summer
495.58µm

9. Oiartzun estruary
early summer
512.07µm

10.Oiartzun estuary
late summer
524.23µm



11. Oiartzun estuary
early summer
573.03µm



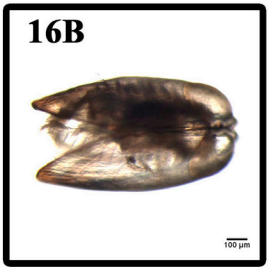
12. Mutriku coast
early summer
647.97µm

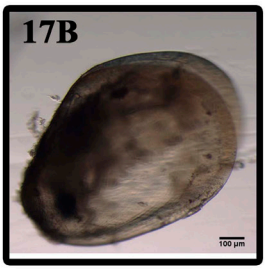
13. Mutriku coast
early summer
649.88µm

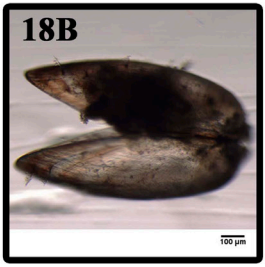
14. Oiartzun estuary
early summer
838.31µm

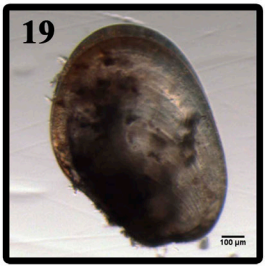
15. Oiartzun estuary
early summer
869.25µm

16. Oiartzun estuary
early summer
872.81µm









17. Oiartzun estuary early summer 952.16µm

18. Oiartzun estuary early summer 989.54µm

POST-LARVA:

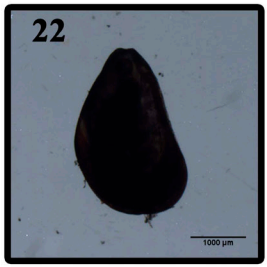
19. Oiartzun estuary early summer 1003.38µm

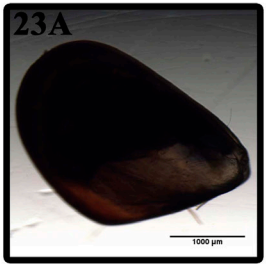
20. Nerbioi coast early summer 1069.34µm

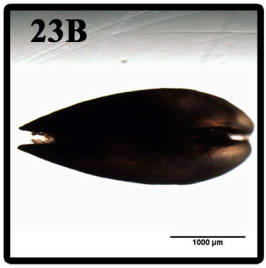
21. Oiartzun estuary early summer 1550.34µm

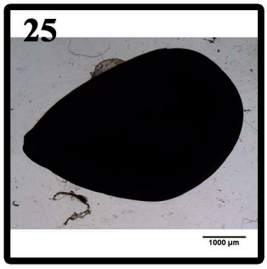
22. Bidasoa estuary early summer 3039.04µm

23. Oiartzun estuary early summer 3283.74µm









24. Bidasoa estuary early summer 4717.89µm

25. Bidasoa estuary early summer 5200.21µm

26. Oiartzun estuary late summer 5400µm

27. Oiartzun estuary late summer 5440µm

28. Oiartzun estuary late summer 8350µm

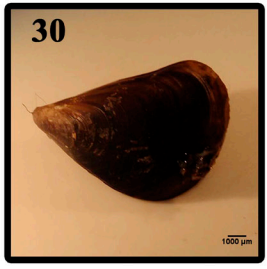
29. Oiartzun estuary late summer 9970µm

30. Oiartzun estuary winter 10590µm

31.Oiartzun estuary late summer 11480µm

32.Oiartzun estuary late summer 13060µm

33. Oiartzun estuary late summer 15450µm



DNA extraction and PCR

All the individuals used in the identification chart showed a clear specific single band of 126 bp, representative of the amplification of the non-repetitive region of the adhesive protein gene (Glu50) from *Mytilus galloprovincialis* Lmk (Figure 3).

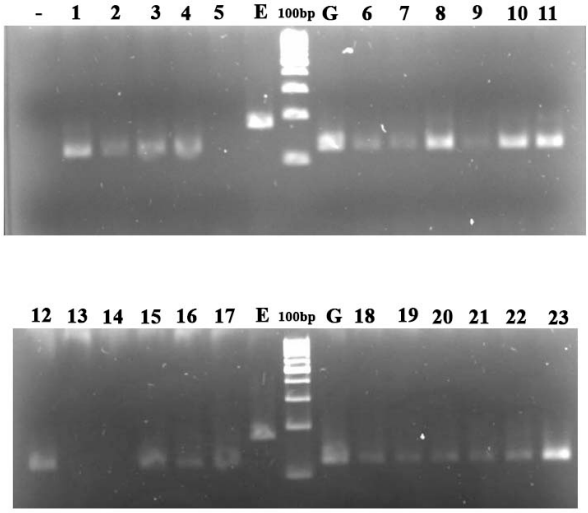


Figure 3 *Agarose gel electrophoresis of PCR products from GLu50 gen (Inoue et al. 1995) of mussel larvae from settlement study. The PCR product of Mytilus edulis L. is 180bp and the PCR product for Mytilus galloprovincialis* Lmk. *is 126bp. The sources of the DNA in lines are as follow: line -: negative control; line 1 to 23: mussel larvae settled on collector ropes at the studied sites along the Basque Coast; Line E: Mytilus edulis; line 100bp: molecular size marker; Line G: Mytilus galloprovincialis*

Spatial and temporal variability in settlement pattern

Mussel size classes found on ropes at each seasonal sampling were not independent of each other. Moreover, as post-settlement processes can affect the observed settlement patterns, the study first focuses on the number of primary settlers (<0.5mm shell length) per meter (Table 2) and on the total accumulated number of spats (primary plus secondary settlers) per meter (Table 3).

Primary settlers

Primary settlers were observed in all sites and on every collection date in each year, but their settlement patterns were quite disparate among the five studied sites (Figure 4). Along the Basque coast, settlement of primary settlers was significantly seasonal (Two-way ANOVA; $p<0.000$) and the number of primary settlers was significantly different in each studied year (Two-way ANOVA; $p=0.043$). Nevertheless, no significant interaction (Two-way ANOVA; $p=0.355$) was observed among seasons in each analyzed year. The factor of season explained 16.9% of the variability in the number of spats settled on ropes, whereas the factor year explained just 3%.

The mean seasonal patterns of primary settlers on suspended ropes along the Basque coast indicated lowest settlement levels in spring and

early summer (Tukey's *test*; p=0.943). Subsequently, a marked increase was observed in *late* summer (Tukey's *test*; p=0.003) and the maximum number of mussels of this shell length class was reached in fall (Tukey's *test*; p=0.999). Thereafter, a decrease (Tukey's *test*; p=0.030) in settlement was observed in winter to an intermediate settlement level.

Regarding annual differences, a higher mean settlement rate was observed along the Basque coast in the second studied year than in the first studied year, especially in spring sampling (Figure 4).

Regarding spatial patterns, significant differences among studied sites were observed in both years (Kruskall Wallis *test*; p<0.000). Results (Table 2) indicated highest settlement of primary settlers in the eastern studied estuarine sites (Bidasoa and Oiartzun estuaries) and lowest settlement in the western studied estuary (Nerbioi estuary). Regarding studied coastal sites, results indicated intermediate settlement in these sites (Nerbioi and Mutriku coasts) compared to estuarine sites.

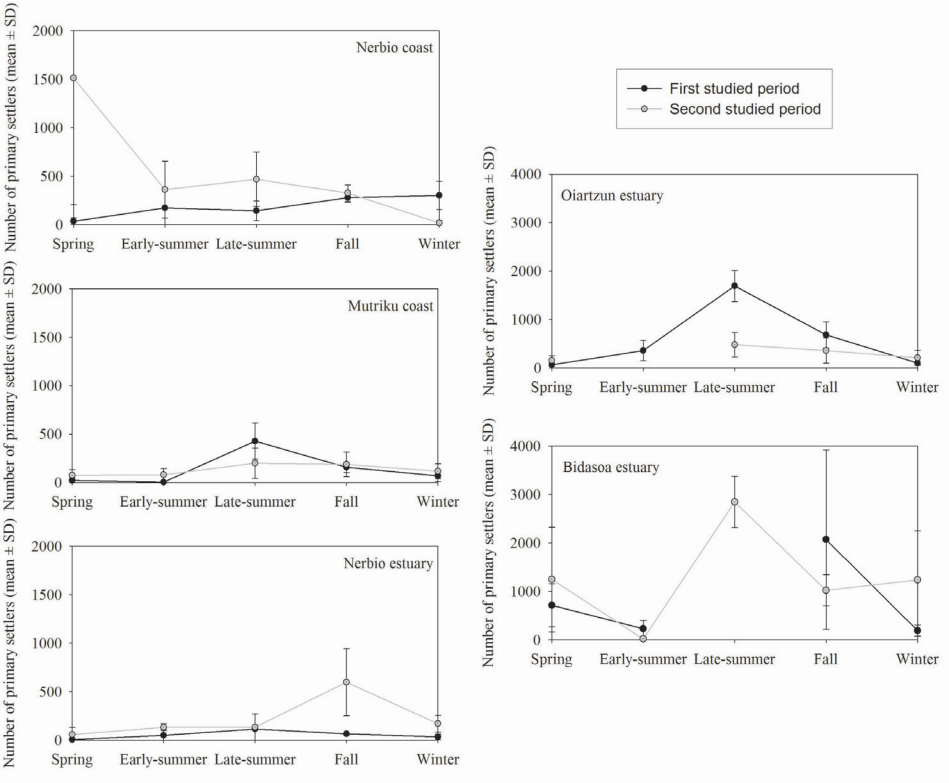


Figure 4 *The number of primary settlers (<0.5mm) (mean ± SD) observed on collector ropes deployed in the studied sites and sampled seasons, in the two separate periods of 10 months each. The first from April 2010 to February 2011 and the second from April 2011 to February 2012*

Table 2 Total number of primary settlers (<0.5mm) *Mytilus* galloprovincilais Lmk. (mean ± SD ind/meter) observed in collector ropes at studied sites and sampled seasons, in the two studied periods of 10 months each. The first from April 2010 to February 2011, and the second from April 2011 to February 2012.

Studied Period	Season	Nerbioi coast	Nerbioi estuary	Mutriku coast	Oiartzun estuary	Bidasoa estuary
First	Spring	35 ± 37	4 ± 8	23 ± 28	65 ± 64	712 ± 442
	Early summer	173 ± 181	48 ± 60	4 ± 7	358 ± 211	229 ± 170
	Late summer	143 ± 101	113 ± 155	428 ± 186	1691 ± 320	
	Fall	278 ± 49	64 ± 16	158 ± 53	683 ± 268	2069 ± 1851
	Winter	301 ± 147	32 ± 28	70 ± 61	100 ± 35	190 ± 118
Second	Spring	1513 ± 1307	56 ± 73	75 ± 58	153 ± 98	1247 ± 1080
	Early summer	362 ± 295	131 ± 35	81 ± 65		20 ± 35
	Late summer	468 ± 282	132 ± 11	199 ± 157	479 ± 250	2846 ± 528
	Fall	326 ± 86	596 ± 347	187 ± 127	359 ± 259	1022 ± 322
	Winter	16 ± 28	168 ± 87	116 ± 78	209 ± 157	1236 ± 1016

Accumulated settlement

The total number (mean ± SD) of mussels obtained from the rope collectors throughout the study are shown in Table 3.

Regarding the pattern in the mean number of accumulated mussels per meter on collector ropes along the Basque coast, results were similar to those patterns observed in primary settlers. Significant seasonal differences were observed (Two-way ANOVA; $p=0.005$), accumulated settlement was significantly different between years (Two-way ANOVA; $p<0.000$) and no interaction was observed (Two-way ANOVA; $p=0.150$) between seasons in each studied year. The factor of season explained 18.1% of the variability in the number of spats settled on ropes, whereas the factor year explained just 5.6%.

Regarding the seasonal pattern of accumulated settlement on suspended ropes along the Basque coast, a low settlement was observed in spring (Table 3). Thereafter, in *early* summer the accumulated number was slightly higher than in spring (Tukey's *test*; $p=0.278$). Subsequently, a considerable increase in the number of accumulated mussels was observed in *late* summer (Tukey's *test*; $p=0.089$). While the number of primary settles was at a maximum in fall, the number of accumulated settlers was at a maximum in *late* summer. This number decreased slightly from *late* summer to fall (Tukey's test; $p=0.995$) but

Table 3 Total number of accumulated settlers (primary and secondary settlers) (mean ± SD ind/meter) *Mytilus* galloprovincilais Lmk. observed in collector ropes at studied sites and sampled seasons, in the two studied periods of 10 months each. The first from April 2010 to February 2011, and the second from April 2011 to February 2012.

Studied Period	Season	Nerbioi coast	Nerbioi estuary	Mutriku coast	Oiartzun estuary	Bidasoa estuary
First	Spring	70 ± 64	4 ± 8	72 ± 101	127 ± 69	832 ± 425
	Early summer	435 ± 445	62 ± 71	94 ± 37	511 ± 284	529 ± 95
	Late summer	232 ± 132	287 ± 241	1089 ± 551	3884 ± 1707	
	Fall	573 ± 76	263 ± 42	571 ± 434	1318 ± 286	4453 ± 3557
	Winter	485 ± 274	113 ± 139	162 ± 200	537 ± 386	890 ± 619
Second	Spring	1825 ± 1462	158 ± 226	169 ± 196	289 ± 142	1828 ± 1798
	Early summer	3607 ± 1885	384 ± 63	171 ± 119		1421 ± 880
	Late summer	1951 ± 834	540 ± 189	452 ± 334	1296 ± 493	6025 ± 1571
	Fall	818 ± 271	1396 ± 501	271 ± 181	549 ± 473	1920 ± 862
	Winter	24 ± 42	450 ± 171	256 ± 91	751 ± 571	2632 ± 2065

still remained high in fall. Levels then decreased significantly from fall to winter (Tukey's test; p=0.036).

Regarding differences between the studied periods, higher levels of accumulated settlement were observed in the second studied year than in the first studied year (Table 3).

Regarding the spatial pattern in the mean number of accumulated mussels per meter on collector ropes, results were similar to those patterns observed in primary settlers. Accumulated settlement was significantly different among studied sites in both studied periods (Kruskal Wallis; p<0.000). Eastern estuaries showed higher accumulated settlement rates than the western estuary, and coastal sites showed intermediate accumulated settlement levels (Table 3) compared to estuarine sites.

DISCUSSION

Spatial and temporal variability in settlement patterns

Mussels are planktotrophic. Their reproductive strategy involves high fecundity, the production of a large number of small eggs, and that their pelagic larvae feed on the available phytoplankton. This high abundance of larvae production is balanced with a high larval mortality (Bayne 1976). Planktonic mortality, as described by Young *et al.* (1996), is

related to fertilization failure (sperm dispersal), extreme temperature and salinity, inadequate food, predation and to the inability to find a suitable substratum. Predators, like many filter feeders, can filter off and ingest larvae, even larvae from their own specie (Kaustky 1982). In addition to the environmental conditions experienced by the larvae, hydrographic conditions can limit the supply of spats reaching the coast (Peteiro *et al.,* 2007) through excessive dispersal. Larvae could be dispersed to areas where no suitable sites for post-larval survival exist (Bayne 1976). In this case, the pelagic phase of *Mytilus* larvae can be prolonged for more than six months (Lane *et al.* 1985).

In this study no planktonic samples were analyzed to measure the abundance of larvae on water column. Bayne (1965) described planktonic mortality rates of free-swimming larvae to be considerable, possibly approaching 99% (Kaustky 1982). It is therefore possible that mortality in the studied area could be considerable as the resultant wild mussel populations along the coast are not very large. The largest population analyzed was from Bidasoa estuary.

Settlement monitoring design was identical and simultaneous in each studied site. Ropes were deployed for 1 month prior to sampling and thus, the formation of a biofilm was possible. Under natural conditions, biofilm formation can occur on collector ropes. The biofilm starts with

the formation of an organic film, made of amino acids, glycoproteins and humic materials. This organic film formation is followed by colonization of bacteria, diatoms, fungi and protozoa (Characklis and Cooksey 1983), which enhances larval settlement on ropes.

A direct relationship between the timing of spawning and subsequent settlement of post-larvae juveniles has been shown for several mussel species (Fuentes and Molares 1994; Alfaro and Jeffs 2003). *Mytilus galloprovincilais* Lmk. at intermediate latitudes can release and fertilize gametes throughout the year. Nevertheless, on the Basque coast massive spawning is concentrated between spring and *late* summer (Ortiz-Zarragoitia *et al.* 2011; chapter 2). Settlement peaks can be expected 1 month after spawning (Aguirre 1979).

The time lag between samplings was quite prolonged in this study and therefore settlement peaks could not be detected precisely. Nevertheless, the temporal settlement patterns observed may be characterized by several peaks. Settlement peaks are usually related to processes such as a synchronized spawning of adults in mussel populations, and also a subsequent and simultaneous development of resultant fertilized gametes. Similarly, gametes being affected by the same transport episodes causes a massive and simultaneous arrival of larvae to specific sites on the coast. Settlement peaks can be related to

one or more of these processes (Pineda 2000).

In the present study, primary spats were present throughout the year, and high abundance was observed from *early* summer to fall. This is consistent with the aforementioned presence of spawning individuals throughout the year and the occurrence of a major spawning period on the Basque coast, as described by (Ortiz-Zarragoitia *et al.,* 2011; chapter 2) from spring to *late* summer. The highest abundance of primary settlers was observed in fall, which suggests that the major spawning peak in the Basque coast is in *late* summer. Nevertheless, it is difficult to confirm the occurrence of a major spawning peak indirectly with recruitment data showing such an extended settlement period, from spring to winter.

Hence, in the present study settlement could be considered protracted. Prolonged settlement can be advantageous for invasive species such as *Mytilus galloprovincialis* Lmk. (Bownes and McQuaid 2009). Indeed, a prolonged settlement period under different environmental conditions has been repeatedly observed in invasive individuals inhabiting different geographical areas (Crawley *et al.* 1986).

The observed number of mussels on collector ropes emphasized spatio-temporal variations of settlement in the studied sites, which may partially be explained by the environmental fluctuations observed on the Basque coast in the studied period (chapter 2). Greater mussel settlement

was found on ropes from estuarine sites compared to coastal sites. In general, these differences in settlement may be primarily attributed to differences in the water circulation pattern and local environmental conditions, as well as to differences in the spawning period between these estuarine and coastal sites.

This higher settlement inside bays and in estuaries may be due to the greater level of accumulation and retention of biological material in geographically confined areas, compared to highly energetic open environments (McQuaid and Phillips 2006). In addition to lower retention, larval dispersal as well as mortality may be higher in open water, which results in heavier recruitment losses on the open coast compared to estuaries (McQuaid and Phillips 2006). When comparing areas inside large estuaries of Galicia (i.e., the SW Bay of Biscay) Peteiro *et al.* (2007) observed greater larval settlement densities in seaward areas. These sites were characterized with higher current velocities and higher turbulence, than inside areas. The largest estuary in the present study was Nerbioi river, whose coastal and estuarine sites have been analyzed. Nerbio coast presented a higher number of settlers than Nerbio estuary, which is likely to be related to the higher water movement at the coastal site compared to the estuarine site.

The spatial differences in settlement could also be attributed to

other differences in local environmental conditions, such as food and temperature. Regarding food, insufficient food may cause starvation or slow growth rates, thus increasing pelagic phase of larvae and resulting in longer exposure to mortality risk (Young *et al.* 1996). Moreover, food supply determines the reproductive cycle of *Mytilus* (Seed and Suchanek 1992). In this study, to measure food supply, the concentration of chlorophyll-a (Chl-a) and POM in the water column was used. When water temperature is above 10ºC and food is available, spat growth is high (Karayücel *et al.* 2002). Temperatures above 10ºC were observed from late winter to fall and corresponded with a high number of both primary settlers and accumulated settlement density. Moreover, Chl-a concentration was highest in April/May, which suggests that nutrients were not a limiting factor for microalgal growth when primary settlers were on the water column. In estuaries higher Chl-a was found in summer, which may have been one cause for the higher settlement in estuaries compared to coastal sites. Similarly, the spawning period differences observed at coastal and estuarine sites, are indeed the result of the environmental variability (i.e., temperature pattern differences and different nutrient seasonality) encountered at estuarine and coastal sites.

Regarding temperature, Cáceres-Martínez and Figueras (1998)b

observed that Galician spatfalls (i.e. the SW Bay of Biscay) may result from the water temperature reaching 12-14°C in March/April in Spain. This increase in temperature was observed in late winter-spring, in the studied sites. Depending on variations in the warming of the water, the spatial pattern of spawning time can vary, as observed in this study, when comparing estuarine and coastal sites (chapter 2). Estuarine sites reached temperatures above 10-12°C before coastal sites. Despite these differences in timing between sites, within each population spawning was coordinated. After spawning, however, coordination between individuals of a population is no longer needed and the duration of the pelagic stage is more variable. Owing to currents, these larvae from different sites may mix, and heterogeneous populations of larvae of different ages will be produced (Kaustky 1982).

This mixed population may settle inshore and post-settlement processes may then define final recruitment on the coast. In the present study, the primary settlers observed from *early* summer to fall could correspond to a local spawning period, and larvae would therefore have spent a normal pelagic phase (1 month; Aguirre 1979). Primary settlers observed in spring and in winter, however, may well have come from a prolonged or delayed spawning population, or from a prolonged free-swimming larvae phase (Karayücel *et al.* 2002).

Suboptimum temperatures and salinities may slow the development of larvae (Bayne 1965). This suboptimal temperature and salinity, however, is unlikely to cause direct mortality, unless it exceeds the extreme levels that larvae can withstand (Young *et al.,* 1996). Water temperature in summer (explained in detail in chapter 2) may have affected the survival of larvae at the studied estuarine sites, particularly in Bidasoa estuary where salinity drops may also have coincided with suboptimal temperatures. Despite these limitations, on collectors deployed at Bidasoa estuary settlement was most favorable and recruitment was more protracted than at the other studied sites. During the second studied period lower salinity levels were observed at Bidasoa estuary and settlement was even higher than in the previous year.

This higher settlement in the second studied period could be related to the colder winter observed in Bidasoa estuary in the first studied period compared to the rest of the studied sites. While suboptimal temperatures can be detrimental for larvae survival, cold winters may enhance final recruitment of larvae on the Basque coast. The cold not only reduces or delays predation (Beukema 1991), but it also reduces the maintenance metabolism of adult mussels. Hence, mussels can use more energy reserves for gametogenesis (Dare and Walker 1992).

The interaction between these environmental factors (temperature,

hydrographic conditions and food availability) may change the quantity or seasonality of the spawning. The observed change in the annual magnitude and seasonality of spawning (chapter 2) may have affected the timing of larvae metamorphosis as suggested by Bayne, 1965; Seed and Suchanek (1992) and would therefore have changed the settlement period.

In the second studied period spawning seemed to start earlier, as the number of primary settlers observed in spring was higher than in the previous spring. Water temperature warmed earlier in the year, with the exception of Bidasoa estuary. In this second studied period, the number of primary settlers decreased later, in *early* summer, and reached a maximum in *late* summer. In the first studied period there was not such a decrease in *early* summer and further, the highest number of primary settlers was observed in fall, not in *late* summer. These changes in spawning may have contributed to the higher observed accumulated settlement in this second studied period.

Mussel competence can be defined as "the capability of a developing individual to initiate settlement and complete morphogenic transformation associated with metamorphosis" (Bishop *et al.* 2006). Indeed, the amount of spatfall on collectors or wild populations is an important aspect for the management of an exploited stock as well as

for the management of parental wild populations (Chavaud *et al.* 1996).

Primary settlement is often a strong determinant of the structure of the adult community in a specific site. Nevertheless, the abundance of primary settlers can differ from final recruitment due to the dispersal and the resettlement of post-larvae stages (Bownes and McQuaid 2009), as well as to the intensity and spatial variation of early post-settlement mortality (Keough and Downes 1982) or, indeed, to the interaction between these factors (Peterio *et al.* 2007).

These mortality rates are also determined by the same local environmental fluctuations that affect settlement. These include fluctuations in variables such as hydrography (McQuaid and Phillips 2000), food availability (Alfaro *et al.* 2006a) and predation (Peteriro *et al.* 2007). Therefore, recruitment was not only influenced by settlement but also by the interaction of local environmental constraints (biological and physical) and the intra-specific competition associated with these environmental limitations (Peteiro *et al.* 2006).

A shortcoming of the method used for settlement assessment (no replacements of ropes and long lag time between samplings) in this study is that it cannot distinguish between mortality and emigration of mussels from ropes, nor can it account for immigration of larger individuals (Bownes and McQuaid 2009). Despite these disadvantages of

the experimental design, valuable information was inferred about these post-settlement processes. The number of accumulated spats on ropes was highest at the end of summer. Thereafter, the number of mussels declined considerably in fall and winter. This marked decrease observed is likely to have been the result of the competition for space between growing mussels and consequential migration of secondary settlers, or the result of predation.

A seed density of over 1200 individuals per meter is necessary to produce high yields of cultured mussels (Okumus, 1993; Cáceres-Martínez and Figueras, 1998b). Extremely high densities, however, may limit growth due to overcrowding and competition for available food (Karayücel 2002). Mussel accumulated densities were slightly lower to those observed in most temperate shores (Okumus 1993; Cáceres-Martínez and Figueras 1998b). In addition, there was a high loss of mussel seeds in winter in both studied years. During fall, when mussel seeds are normally gathered for grow-out sites at industrial cultivation, local hydrodynamics and disturbance regimes, such as the greater number of storms, may have affected secondary relocation and the settlement of post-larvae. Local hydrodynamics, however, were not analyzed in this study.

Mussels are most sensitive to this overcrowding when newly

settled and still actively creeping. During this phase pediveliger larvae, if disturbed, are able to detach from ropes and swim or drift away and reattach to another surface (Bayne 1965; Cáceres-Martínez *et al.* 1993). This can happen several times during this phase. The scarcity of the post-larvae of >3mm on the ropes in winter in this study suggest that the majority of young post-larvae mussels may have detached. Hence, it is suggested that ropes be collected earlier. Above this shell length, mussels can increase their byssus production, which allows them to attach more efficiently and to keep in contact with the rope using their long byssus threads. These long threads allow mussel populations on ropes to grow outwards from the centre (Kautsky 1982).

Fish predation was not analyzed in this study, however, it may have been another important cause of the observed loss of mussels. Indeed, fish predation is considered as a major cause of seed mortality in industrial cultivation (Schiel *et al.* 2004; Rilov and Schiel 2006).

Conclusions

The present study is the first to investigate mussel settlement patterns on suspended collector ropes along the Basque coast and indicates that ropes may be a useful way of monitoring and perhaps predicting spatfalls.

One of the main goals of this study was to asses if recruitment

was more affected by settlement itself or by post-settlement processes. The results of this study shed light on the trends and features of spatial variability in the seasonal patterns of larvae settlement and recruitment of *Mytilus galloprovincialis* Lmk. along the Basque coast. Results showed that recruitment on the Basque coast was more determined by post-settlement processes than by settlement abundance.

More research is necessary to identify sites of optimal mussel settlement on the Basque coast. Nevertheless, statistical analyses were conclusive and higher recruitment rates were observed at estuarine sites compared to coastal sites. Particularly, recruitment was higher in the western estuaries, Oiartzun and Bidasoa. This suggests that seeds collected at these sites may serve to supply grow-out sites in the open ocean. Therefore, these seeds may fulfill future seed demand for commercial purposes.

The carrying capacity of these sites was not analyzed in this study and thus, the total number of mussel seeds that could be gathered at these sites is not yet known. This may be an area of future research. The Basque coast suffers from user conflict, and the allocation of areas for seed gathering may be limited. Despite these limitations, if these sites are to be used to gather seeds, care should be taken as to when to harvest. As such, ropes could be transferred in *late* summer-fall to aquaculture

facilities where post-settlement processes could be better controlled. Thus, final productivity of ropes may be improved and the considerable loss in seed production observed in fall-winter will be reduced.

Further studies with different collector types, different collector lengths and different deployment depths, detailed hydrographical conditions (wave, current, plankton community, etc.), as well as possible predation rate are necessary to understand the observed settlement and recruitment on the Basque coast in order to create successful mussel aquaculture in the future.

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CHAPTER 4

GROWTH, BIOCHEMICAL PROFILE, AND FATTY ACID COMPOSITION OF MUSSEL (*Mytilus galloprovincialis* Lmk.) CULTURED IN THE OPEN OCEAN OF THE SE BAY OF BISCAY (BASQUE COUNTRY).

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SUMMARY

Growth performance, allometry, condition index, and biochemistry of mussel (*Mytilus galloprovincialis* Lmk.) cultured in a submerged longline system, located in the SE Bay of Biscay, were investigated from June 2013 to August 2014. Mussels grew faster from spring to autumn than in winter. Chlorophyll-a concentration along with sea current speed will likely be major factors controlling mussel growth and survival success in the area of study. The lowest condition indices and the highest ash contents were respectively found during winter; so mussels cultured herein would surely reach the most suitable conditions for harvesting and commercialization within autumn, spring and summer seasons. The positive growth and low chlorophyll-a concentrations found during summer may indicate utilization of a non-phytoplankton food source by mussels. There was no clear evidences for a depth associated physiological change on the mussel general performance. This contribution represents the first available information on mussel biology and open ocean aquaculture from the Bay of Biscay.

keywords: Mussel; *Mytilus galloprovincialis* Lmk.; Open Ocean; Growth; Condition index; Nutritional quality; Proximate composition.

INTRODUCTION

Blue Growth is a generally accepted long-term strategy to support sustainable growth in the marine and maritime sectors. Farming in open ocean waters has been identified as one potential option to increase seafood production, and it has been a focus of international attention for more than a decade (Bridger and Costa-Pierce, 2003).

Shellfish species (mainly mollusk bivalves) have also been considered attractive candidates for open ocean farming worldwide (Buck *et al.,* 2005; Buck, 2007; Langan, 2000). These species have been an important food source in the socioeconomic context of the SE Bay of Biscay (Gracia, 1996). However, during recent decades, all the mollusks (especially mussels) consumed in the region are imported. Albeit, aquaculture production of bivalve species is well established and occurs in many countries (Bayne, 1976; Norling and Kautsky, 2007; Rius and Cabral, 2004; Smaal, 1991), this activity has never been developed in the SE Bay of Biscay due to factors like strong hydrodynamic coastal conditions (Galparsoro *et al.,* 2012), water-quality industrial issues on main rivers and estuaries (Chust *et al.,* 2009), and strict regulations on marine habitats monitoring and protection (Borja *et al.,* 2011; Pascual *et al.,* 2011).

Mussels are generally appreciated for their nutritive quality,

organoleptic properties, and economic potential (Bayne, 1976). Albeit mussel shell appearance can be decisive in market prize and purchase motivation (Brenner *et al.,* 2012), product quality is mostly regulated by biochemical composition and condition indices (Filgueira *et al.,* 2006; Fuentes *et al.,* 2009; Orban *et al.,* 2002, 2007).

The Mediterranean mussel (*Mytilus galloprovincialis* Lmk.) is widely distributed around the temperate shelf waters of the Northeast Atlantic Ocean and its adjacent seas, including the southern area of the Bay of Biscay (Garmendia *et al.,* 2011; Gosling, 1992; Marigómez *et al.,* 2007; Martínez-Pita *et al.,* 2012; SanJuan, 1994). *M galloprovincialis* Lmk. can co-occur and hybridize with other mussel taxa (Brenner *et al.,* 2014; Dias *et al.,* 2009) when geographic areas overlap and patchy environments arise (Hilbish *et al.,* 2002). Water temperature and food abundance are the main environmental factors regulating mussel physiology and reproduction (Seed, 1976). Specifically, the interactions among type and abundance of phytoplankton, temperature, salinity, and local hydrodynamics (currents and waves) are expected to modulate the growth rates and the condition index.

Mussel (*Mytilus* spp.) growth and allometry have been extensively studied by many authors throughout the world (e.g., Akester and Martel, 2000; Hosomi, 1985; Lauzon-Guay *et al.,* 2005; Mallet and Carver, 1995;

Pérez-Camacho *et al.*,1995; Seed, 1973; Steffani and Branch, 2003).

During the last decades, no studies have investigated the patterns regulating mussel growth, physiology and/or biochemistry in the open ocean of the present case-study region (i.e., Izagirre *et al.,* 2014; Tamayo *et al.,* 2008). Most of them have been focused on characterization of reproduction cycles (Garmendia *et al.,* 2010; Ogueta *et al.,* 1995; Ortiz-Zarragoitia *et al.,* 2011; Ortiz-Zarragoitia and Cajaraville, 2006, 2010) or mussels ´use as biomarkers of water quality and environmental pollution (Franco *et al.,* 2002; Izagirre and Marigómez, 2009; Jimeno-Romero *et al.,* 2009; Marigómez *et al.,* 2004; Solaun *et al.,* 2013). However, all of these investigations are based on laboratory studies or used natural specimens collected from estuaries or intertidal local populations.

Mussel growth seems to be seasonal, with gross growth concentrated in one season (Seed, 1973). Shell growth, however, appears to be continuous during the year (Stirling and Okumus, 1995). Shell growth can be continuous even in the absence of undernourished feeding, as it is formed mostly from dissolved calcium present in seawater (Alunno-Bruscia, 2001). Amongst other authors, Akester and Martel (2000), Alunno-Bruscia *et al.* (2001) and Seed (1973) already reported that the main factors affecting on mussel shell shape morphology are density, age, wave exposure, mussel size and availability of food.

Mussel physiology and energy balance can also be inferred through the condition of individuals under given environmental scenarios (Lucas and Beninger, 1985). Similarly, the condition index in mussels is mostly affected by population density, size, gonad development, salinity, temperature, food supply, and environmental contaminants at seasonal level (Bayne and Worrall, 1980; Lucas and Beninger, 1985; Okumuş and Stirling, 1998; Orban *et al.,* 2002; Rhoads and Lutz, 1980).

Meat quality at a particular time can reflect the composition of the daily diet of mussels, which consisting of phytoplankton (e.g., diatoms, flagellates), bacterioplankton, organic detritus, and micro-zooplankton (Alkanani *et al.,* 2007; Hawkins and Bayne,1991), may vary with daily temperature, water stratification, and hydrodynamics (Cartier *et al.,* 2004). Similarly, the proximate composition of mussels undergoes a seasonal cycle characterized by phases of accumulation and depletion of reserves, reflecting the gonad development stage (Orban *et al.,* 2002) as well as the aforementioned food availability. Nevertheless, mussel species are able to survive long periods of starvation, adjusting their metabolism to changing environments (Hernandez *et al.,* 2013).

Both temperature and food abundance regulate gametogenic cycle and associated storages of glycogen, proteins, and lipids (Gabbot and Bayne, 1973; Seed, 1976). Glycogen level represents a suitable indicator

of condition related to gonad cycle (Gabbott, 1975), whereas protein content represents growth (Ojea *et al.,* 2004). Similarly, increases in the lipid content have been reported to indicate proximity to spawning (Prato *et al.,* 2010) and favorable conditions of phytoplankton in the local environment (Freites *et al.,* 2002a). Lipids in mussels are known to contain a wide variety of structural fatty acids, including saturated, SAFAs, monounsaturated, MUFAs, and polyunsaturated, PUFAs (Martínez-Pita *et al.,* 2012). Variations in the total fatty acid composition of mussels (*Mytilus galloprovincialis* Lmk.) are reported to have both endogenous and exogenous origin, and depend on multiple aspects such as season, origin, zone of culture, reproductive status, temperature, and the phytoplankton profile in the diet (Dridi *et al.,* 2007; Fernández-Reiriz, 1989; Fuentes *et al.,* 2009; Ojea *et al.,* 2004; Prato *et al.,* 2010). DHA (22:6 ω3) is known for promoting growth in shellfish (Parrish, 2013) and particularly plays an important role at the structural and functional levels of cell membranes involved in oogenesis, embryogenesis, and larval survival (Martínez-Pita *et al.,* 2012). EPA (20:5 ω3) has been determined to have an energetic function. It acts as an energy source during gametogenesis until the end of embryogenesis (Martínez-Pita *et al.,* 2012; Sánchez-Lazo *et al.,* 2012). There are a number of ω6 long-chain PUFA commonly found in mussels which may be nutritionally

important.

The main objectives of the present study were to evaluate: (i) growth; (ii) condition index; (iii) shell shape; (iv) proximate composition; and (iv) fatty acid profile in mussels (*Mytilus galloprovincialis* Lmk.) cultured at the open ocean conditions of the SE Bay of Biscay. The study aims to provide new biological information of use for the potential aquaculture development of this specie within the case-study region.

MATHERIAL AND METHODS

Experimental site

The Basque coast is located in the SE Bay of Biscay (Figure 1.A. and Figure 1.B.). It extends along 150 km and is oriented E-W (Figure 1). The climate is temperate, oceanic, with moderate winters and warm summers (Collins and Borja, 2004). An open ocean experimental site (43° 21.39' N, 2° 26.90' W), located at 2 miles off the coast (Figure 1.C. and Figure 1.D.) and fulfilling the wave energy criteria described by Ryan (2004), was established by the regional authorities aiming to develop further aquaculture activities in the region.

A suspension shellfish culture system based on a conventional longline (i.e., subsurface structure consisting of anchors and submerged flotation from which mussel ropes can be suspended; Buck, 2007; Langan, 2000) was developed at pilot-scale. The experimental grow-

out ropes system was built employing polyamide and poly-steel ropes suspended in the water column from horizontal headlines at submerged conditions. Grow-out ropes of identical length (12m) were hung from two headline depths, 5m and 15m depths, at a distance of 0.5m between ropes (Figure 1.E.). That range of depths was selected to prevent any effect of the local tidal range influence (Gonzalez *et al.,* 2004).

Environmental conditions

Local environmental conditions on seawater temperature and currents were obtained from the NEMO model (Nucleus European Modelling of the Ocean). Information on waves (i.e., significant wave height, H_s, and peak period, T_p), was obtained from two metocean buoys, called Bilbao-Vizcaya and Matxitxako, owned and maintained by Puertos del Estado and Euskalmet, respectively.

Chlorophyll-a concentration (as indicator of phytoplankton biomass) was measured *in situ* in the water column by means of a Sea-Bird CTD (Conductivity, Temperature and Depth). The CTD fluorescence was calibrated, regularly, with water samples filtered through Whatman GF/C filters and analyzed by spectrophotometry after pigment extraction in acetone. Six field trips were conducted seasonally from March 2013 to August 2014, and continuous vertical profiles were obtained at four stations surrounding the experimental site (Figure 1.C.). During

processing, the data from each corresponding profile were vertically averaged for 5 and 20m depth respectively, to provide a synthesized resolution of the mussel ropes environment (Figure 1.E.).

Growth rate and Condition

The study was carried out from June 2013 to August 2014. *Mytilus galloprovincialis* Lmk. juvenile mussels were sub-tidally collected from natural populations and transferred (by manual seeding) to 24 target growing ropes suspended and distributed (12:12) at two different culture depth levels (5m and 15m depths) from the longline system. The initial mussel mean size and density were 17.40 ± 5.07mm and 681 ± 37 individuals per linear meter. At each sampling time, three mussel ropes from each culture depth level (5m and 15m depths) were randomly sampled every 4-5 months (depending on environmental conditions) and transported to laboratory.

In the laboratory, mussels were detached from ropes. These mussels were then weighed to obtain the total weight on ropes and cleaned ropes were measured to the nearest 0.1cm. Subsequently, a subsample of approximately 15,000g of mussels per individual rope was prepared for analysis. Individual mussels were carefully detached from each other by cutting the byssal threads and cleaned of encrusting organisms. Individuals were then measured and counted to calculate size

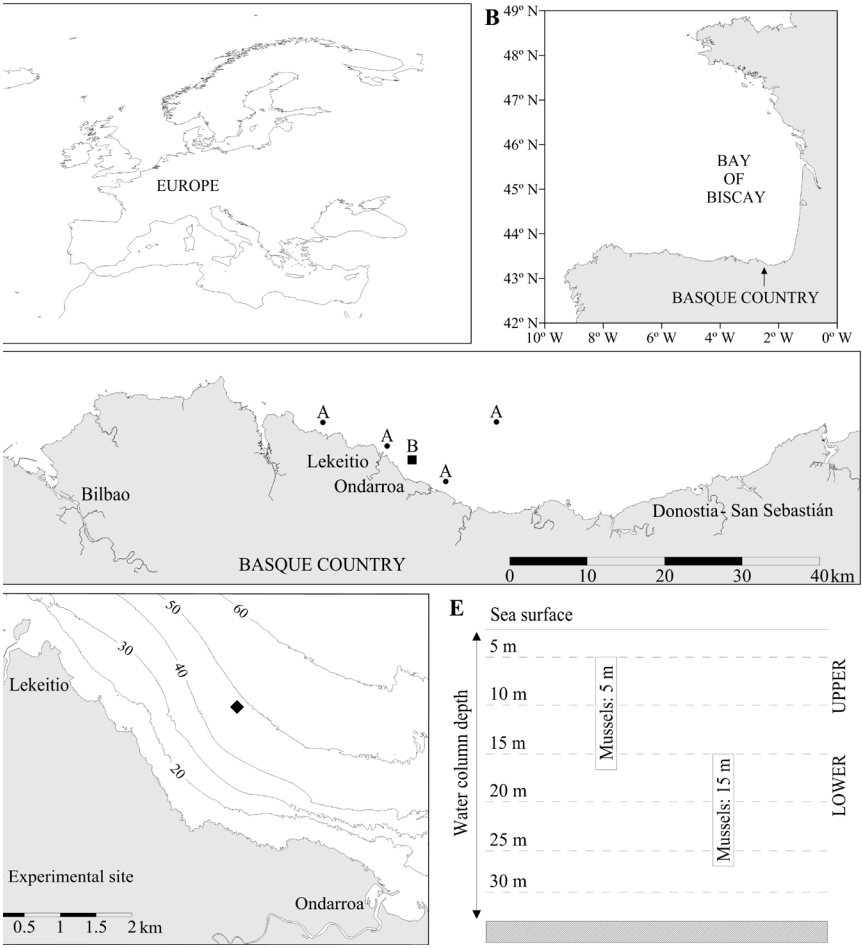

Figure 1:*A) Bay of Biscay location. B) Basque Country coast. C) Map of the study area showing the specific location of the open ocean experimental site (Mendexa:* **B** *black square) and the stations for chlorophyll-a data acquisition (***A** *black points). D) Bathymetry map of the experimental site. E) Synthetic scheme of the suspension range of the mussel culture ropes within the water column at the beginning of the experiment.*

frequency distribution. Individuals of the same size class were weighed together to obtain the total weight on ropes of each size class as well as the mean theoretical individual weight per size class. These data were used for the estimation of density (individual's·m^{-1}) and mortality on ropes. Thereafter, a subsample of 50 mussels, proportional to the size-class frequencies found in each rope and depth level, was considered for morphometric analysis. The individuals were measured for their shell length (L; maximum anterior–posterior axis), shell height (H; maximum dorso-ventral axis), and shell width (W; maximum lateral axis) using a digital caliper to the nearest 0.1mm.

Similarly, the subsample of 50 mussels, was removed for condition index determination. Mussel shell and associated meat were carefully dissected away, separately placed in pre-weighed porcelain trays for drying at 80°C for 24 h and then weighted to the nearest 0.001g (Walne, 1976). Subsequently, dry mussel meat was individually ashed at 450°C for 4 h in a muffle furnace (Brake *et al.,* 2004), cooled in a desiccator and weighted again to determine the ash-free dry weight content (Lucas and Beninger, 1985).

Biochemical composition and fatty acids

Another subsample of 50 mussels, proportional to the size-class frequencies found in each rope, was collected for biochemical

characterization at each sampling time and culture depth level. Three pooled samples (of 50 individuals per rope and depth level) were prepared in the laboratory and minced in a food processor (IKA® M 20 universal mill, IKA 1603601, Germany). Then, the samples were analyzed for moisture and ash according to gravimetric methods and total protein determined according to Kjeldahl ś method (AOAC, 1975). Samples were digested in a digestor BUCHI B-435, distilled using a BUCHI B-324 distillation unit and automatic volumetric titration developed through a Mettler Toledo T- 50 (Mettler Toledo, United States). Finally, glycogen concentration ($mg \cdot g^{-1}$ mussels' tissue) was calculated based on a mussel glycogen standard (Sigma, Saint Louis, MO) following the methodology reported by Gallardi *et al.* (2014).

Similarly, a subsample of previously used mussels' tissue pool by rope, was collected for lipids determination at each sampling time and culture depth level. Three pooled samples (of 50 individuals by rope and depth level) were prepared and total mussel lipid (TL) extracted on the basis of the method reported by Blight and Dyer (1959), modified by Hanson and Olley (1963).

Fatty acids were determined from the lipid samples. Fatty acid methyl esters (FAMEs) was separated and identified using an Agilent Technologies 7890 Gas Chromatographer equipped with auto-injector

and a Flame-Ionization Detector (FID) and a DB-WAX 122-7032 Agilent Technologies capillary column (30m_0.25mm I.D.; film 0.25μm). Helium was used as a carrier gas and the initial oven temperature was 50°C for 2 min, followed by an increase at a rate of 5°C·min^{-1} to a final temperature of 240°C for 5 min. The temperature of the inlet was 250°C and the temperature of the detector 290°C. Individual FAMEs were identified by comparison of retention times with four commercial fatty acid standards (PUFA 1, PUFA 3, BAME, and 37-component) supplied by Supelco (Bellefonte, PA) for area percent normalization. Relative quantities were expressed as weight percent of total fatty acids in each sample.

Statistics

Mussel weight-at-length data were fitted through the model reported by Ricker (1979). Instantaneous (G) rate of growth was also derived for each culture depth level group. Daily specific growth rates, in terms of weight, were defined as G (x100%) (Laurence, 1976).

Shell morphology was investigated through the general linear function where y and x are size-related measures, and a and b are constants (Pauly, 1983). After logarithmic transformation of all variables, spatial (depth) and temporal (seasonal) effects were assessed using ANCOVA (Rohlf and Sokal, 1981), with α set at 0.05.

Condition index was calculated as: (i) the ratio of meat dry weight (DMW) to shell weight (SW); and (ii) the ratio of ash free dry weight (AFDW) to shell weight (SW). Both condition indices were calculated following Lucas and Beninger (1985).

All collected environmental and biological data were plotted using Sigmaplot (11.0) statistical and graphical software (Systat software) and statistical analysis was carried out using a statistical package (SPSS 17.0 for Windows®). Data were tested for normality (Shapiro-Wilk test) and variance homogeneity (Leven's test). Spatial (depth) and temporal (seasonal) effects were analyzed using two-way ANOVA for homogenous variances and Tuckey's *test* as post hoc test, or Kruskal-Wallis and Mann-Whitney U *test* for heterogeneous variances. Arcsine transformation (Zar, 1984) was applied to the data expressed as a percentage. The significance level was set at $p = 0.05$.

RESULTS

Environmental conditions

The environmental conditions (temperature, currents, and waves) that characterized the water column are shown in Figure 2. The annual mean time series of temperature (Figure 2.A.) and current speed (Figure 2.B.) did not show significant differences (ANOVA; $p>0.05$) between the two culture depth levels tested. However, both variables showed

statistical differences with time (ANOVA; $p<0.001$). Similarly, statistical differences (ANOVA; $p<0.05$) with time were also observed for H_s (significant wave height) and T_p (peak period) among different seasons (Figure 2.C.).

The mean water temperature ± standard deviation was 16.8 ± 3.2ºC in the 5m depth culture, and 15.8 ± 2.5ºC in the 15m depth culture. The maximum and minimum temperature values were 22.4ºC and 11.8ºC in the shallower culture, and 21.4ºC and 11.8ºC in the deeper culture, respectively. The mean current speed ± standard deviation was 8.7 ± 4.6cm·s^{-1} and 8 ± 4.2cm·s^{-1} at 5m and 15m depths, respectively. The maximum and minimum current speeds were 25.8 and 0.9cm·s^{-1} in the shallower depth, and 27.8 and 0.5cm·s^{-1} in the deeper one. The observed mean values of H_s and T_p ± standard deviation were 2 ± 1.4m and 9 ± 3.6s, respectively. Their maximum and minimum values were 8m and 19s, and 0.4m and 1.5s, respectively

The chlorophyll-a (Chl-a) concentration in the water showed significant differences with sampling time (ANOVA; $p=0.001$) (Figure 3.). Higher chlorophyll-a concentration was found in winter, but no significant differences (ANOVA; $p=0.854$) were found between the two culture depth levels or between the sampling stations when each culture depth level (shallower and deeper cultures) were compared separately

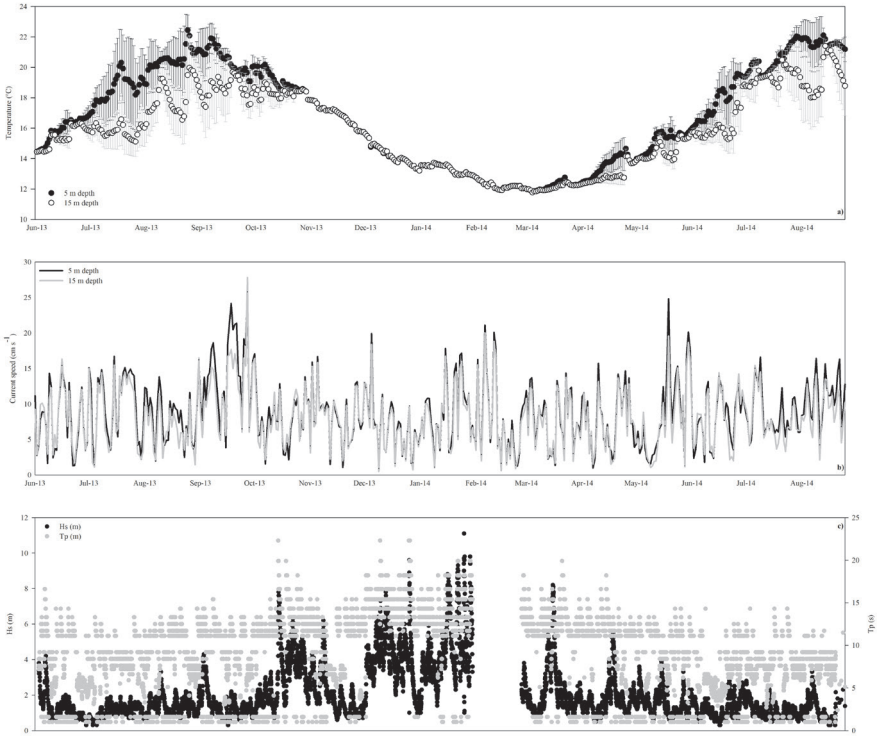

Figure 2 *Daily trends of (A) temperature and (B) current outputs from the NEMO model (http://www.myocean.eu). (C) Time series of the significant wave height, Hs, and peak period, Tp, as measured at the nearest reference buoy (Puertos del Estado) to the experimental site of the present study between June 2013 to August 2014 in the two culture depth levels (5m and 15m depths) tested.*

(ANOVA; p=0.411 and p=0.802, respectively). The mean values of Chl-a at both culture depth levels during the study period was 0.68 ± 0.58μg l^{-1}.

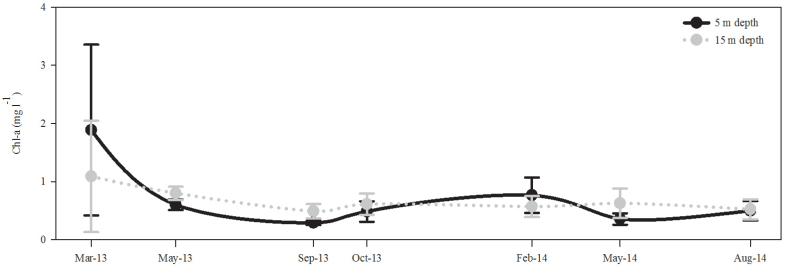

Figure 3 *Temporal distribution of chlorophyll-a concentration (Chl-a) measured at four stations (mean ± SD) in the vicinity of the experimental site. Mean concentrations are shown for two intervals of the water column that are assumed could influence the culture depth levels (5m and 15m depths, 5-20m and 15-30m, respectively).*

Growth rate and condition

The potential equation provided a good fit to the weight-at-length data (Figure 4.A.), explaining between 91 and 89% of the growth variability in mussels cultured at upper and lower depths, respectively. Culture depth significantly affected the weight-at-length relation (ANCOVA; p=0.005). The regression equations were expressed as follows: $DW=0.000231 \cdot L^{2.441}$ (R^2=0.913, n=600, shallower culture), and $DW=0.000282 \cdot L^{2.407}$ (R^2=0.899, n= 600, deeper culture). For each culture depth, the instantaneous and daily specific rates of growth are shown in Table 1. The daily specific growth rate (weight gain) ranged from 0.84 to 0.85% between 5m and 15m depth levels.

The growth in L compared in day units (dd; days post-suspension, see Figure 4.B.) was significantly affected by culture depth (ANCOVA; p=0.001). The corresponding exponential equations were fitted as follows: $L=19.299e^{0,003 \cdot dd}$ (R^2=0.747, n=600, shallower culture), and $L=21.467\, e^{0.003 \cdot dd}$ (R^2=0.618, n=600, deeper culture).

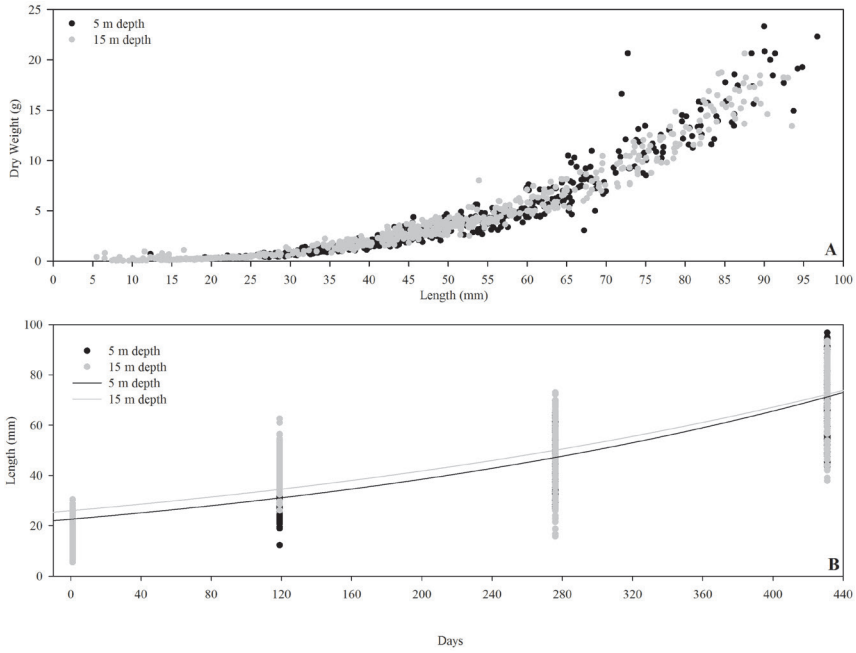

Figure 4 *Mussel (Mytilus galloprovincialis Lmk.) (A) Body´s Dry weight(DW) and length(L) relationship in the upper culture depth range (black points) and in the lower culture depth range (grey points);(B) Growth in length(L) and days(dd) relationships of mussel cultivated in the 5 m depth level L = 19.29e 0.003 dd (R2=0.747, n=600) (black points) and in the 15 m depth level L = 21.467 e0.003 • dd (R2=0.618, n=600) (grey points).*

Table 1 Biometric characteristics of mussels (*Mytilus galloprovincialis* Lmk.) shells by sampling time at both culture depth levels tested. * Statistical significance set up at ($p < 0.05$) (pair-wise comparison).

	G	%
5 m depth		
Length	0.00323	0.32
Shell weight	0.00819	0.82
Dry Meat weight	0.00992	1.00
15 m depth		
Length	0.00323	0.32
Shell weight	0.00832	0.84
Dry Meat weight	0.00941	0.95

SW and DMW

Mussel growth in weight (shell weight (SW) and dry meat weight (DMW)) was significantly affected by culture depth (Mann-Whitney test; p=0.007 and p=0.010, respectively) (Figure 5.A. and 5.B.). SW and DMW also varied significantly with season (Kruskal-Wallis test; p<0.000). The mean SW values ranged from 0.2g to 8.3g and 8.8g between 5m and 15m culture depth levels, respectively. Similarly, the mean DMW values ranged from 0.02g to 1.6g and 1.3g between 5m and 15m culture depth levels, respectively. In fall, significantly higher mean SW and DMW were observed in the lower culture depth (Mann-Whitney test; p<0.000).

Condition indexes

Freeman ś and AFDW condition indices (Figure 5.C. and 5.D.) were affected by sampling time at both culture depths (Kruskal-Wallis *test*; p<0.000). Along the studied period Freeman's condition index was not affected by culture depth (Mann-Whitney; p=0.561), whereas AFDW condition index was affected by culture depth (Mann-Whitney test; p=0.004). Lower values were found in winter (March 2014) and highest mean values of both Freemans ´and AFDW condition indexes were found at 5 m depth level and in summer (August 2014).

Regarding seasonal differences in Freeman's condition index due

to culture depth, significant differences were observed in fall (Mann-Whitney *test*; p=0.002), but not in winter (Mann-Whitney *test*; p=0.871). In summer, significant differences were observed (Mann-Whitney *test*; p<0.000). In October 2013, Freeman's condition index was significantly higher at the 15m culture depth. However, at the end of the study period, in August 2014, significantly lower values were found at the 15m culture depth. Regarding differences in AFDW condition index due to culture depth, the condition index was not affected by culture depth in fall (Mann-Whitney *test*; p=1.138) but was significantly affected in winter (Mann-Whitney *test*; p<0.000). Similarly, significant differences were observed also in summer (Mann-Whitney *test*; p<0.000). In winter and in summer mussels, significantly lower AFDW condition index values were found in the deeper culture mussels.

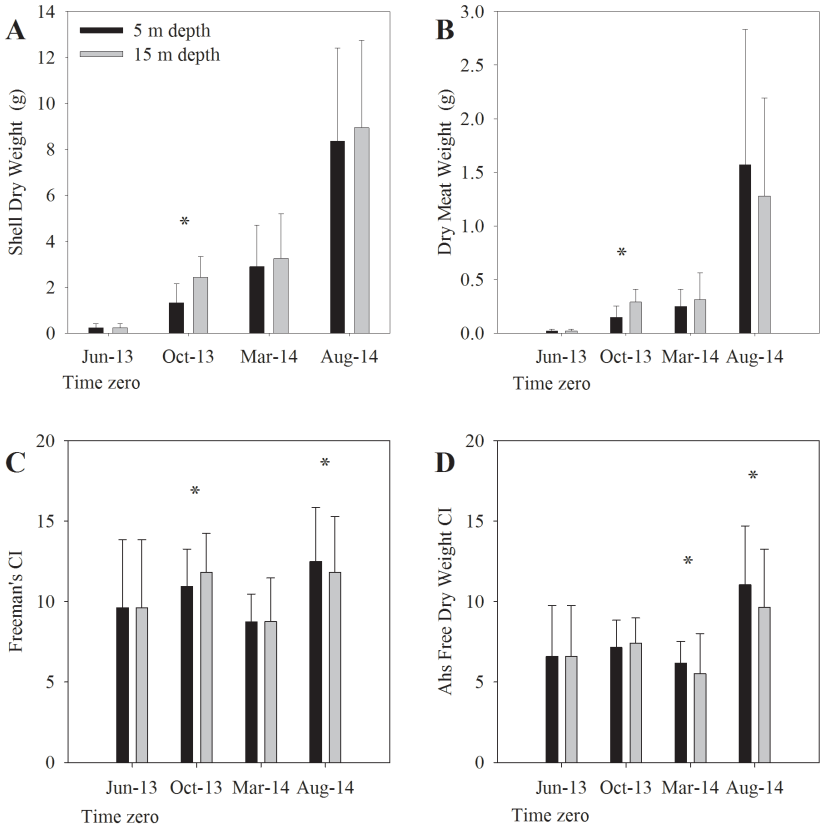

Figure 5 *Change in mean (± SD): (A) Shell weight (SW), (B) Dry meat weight (DMW), (C) Freeman's condition index (CI) and (D) Ash Free dry weight Condition index (AFDW CI). Bars represent mean (± SD) in 5m and in 15m culture depth levels (black and grey bars, respectively). * represents significant Mann-Whitney rank sum test ($p<0.05$).*

Density

The seasonal trends in density are shown in Figure 6. The mussel density was affected by culture depth (t=2.617, d.f.=22, p=0.016). Particularly, density in the shallower culture was significantly higher than at the lower depth in October 2013 (t=3.838, d.f.=4, p=0.018) and in August 2014 (t=4.821, d.f.=4, p=0.009).

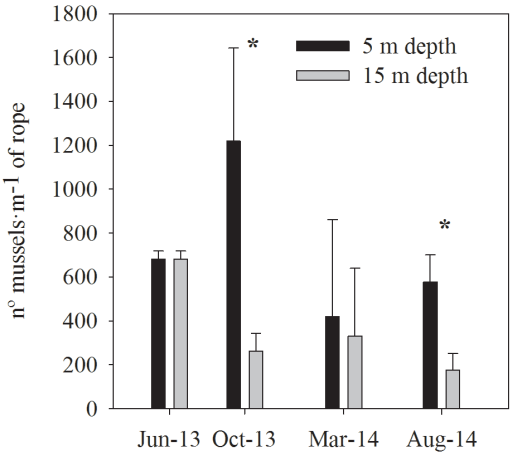

Figure 6 *Density of Mytilus galloprovincialis* Lmk. *Evolution of number of mussels per lineal meter of rope within the two culture depth levels (5m and 15m depths) at each of the sampling time of the present study.* * represents significant pair wise comparison test (p < 0.05).*

Shell shape

Morphometric characteristics of the mussel shells from both culture depths are shown in Table 2. In general, statistical differences between the shell length (L) and shell height (H) of mussels cultured at both culture depth was detected (Mann-Whitney test; p=0.042 and p=0.007). Conversely, no significant differences were detected in shell width (W) between mussels cultured at both culture depths (Mann-Whitney test; p=0.159). As observed, mussel individuals from the lower depth displayed significantly higher shell length (L) and shell height (H) values than those cultured at the lower depth.

Table 2 Biometric characteristics of mussels (*Mytilus galloprovincialis* Lmk.) shells by sampling time at both culture depth levels tested. * Statistical significance set up at ($p < 0.05$) (pair-wise comparison).

	2013		2014	
	June	**October**	**March**	**August**
	Time Zero			
Length (mm)	17.39 ± 5.07	34.94 ± 7.45*	48.97 ± 11.92	69.77 ± 13.53
Height (mm)	9.51 ± 2.73	19.1 ± 3.72*	26.19 ± 6.25	37.07 ± 7.28
Width (mm)	5.92 ± 1.66	11.87 ± 2.95*	16.05 ± 4.19	24.7 ± 10.41
Length (mm)	17.39 ± 5.07	44 ± 6.47*	49.46 ± 13.04	69.77 ± 15.82
Height (mm)	9.51 ± 2.73	24.88 ± 3.36*	26.83 ± 6.87	36.83 ± 7.02
Width (mm)	5.92 ± 1.66	14.38 ± 2.18*	16.08 ± 4.31	23.35 ± 6.16

The general allometric relationships between shell length (mm) and shell height (mm), shell length and shell width (mm), or between shell width (mm) and shell height (mm) of mussels cultured at both culture depths are shown in Table 3 and Figure 7.

Table 3 *Mytilus galloprovincialis* Lmk. Parameter of shell shape (length (L), width (W) and height (H)) regression equation used to relate shell width (mm) or shell height (mm) to shell length (mm), and shell height (mm) to shell width (mm) of all sampled mussels from different culture depth (Upper depth vs. Lower depth). Equation fitted to log10 X = log10 a + b log10 Y. where a intercept and b slope, and X and Y are shell length measurements. Error of correlation coefficient (r), number of specimens (n) used to derive equation are also given.

Sampling date	*Alometric relation*	*Depth*		*B*	*SE*	*t*	*sig*	*95% CI*		R^2	*N*
								Upper	*Lower*		
Total	*L/H*	*5m*	*Intercept*	-.205	.009	-21.735	.000	-.223	-.186	0.989	599
			Slope	.960	.006	161.755	.000	.949	.972		
		15m	*Intercept*	-.178	.013	-13.750	.000	-.203	-.152	0.979	599
			Slope	.948	.008	118.163	.000	.932	.964		
	L/W	*5m*	*Intercept*	-.442	.014	-31.410	.000	-.470	-.414	0.976	599
			Slope	.982	.009	110.642	.000	.964	.999		
		15m	*Intercept*	-.376	.016	-24.018	.000	-.407	-.346	0.969	599
			Slope	.936	.010	96.215	.000	.917	.955		
	W/H	*5m*	*Intercept*	.268	.010	26.858	.000	.249	.288	0.949	599
			Slope	.941	.009	105.993	.000	.924	.959		
		15m	*Intercept*	.241	.010	24.086	.000	.221	.261	0.954	599
			Slope	.979	.009	111.528	.000	.962	.996		

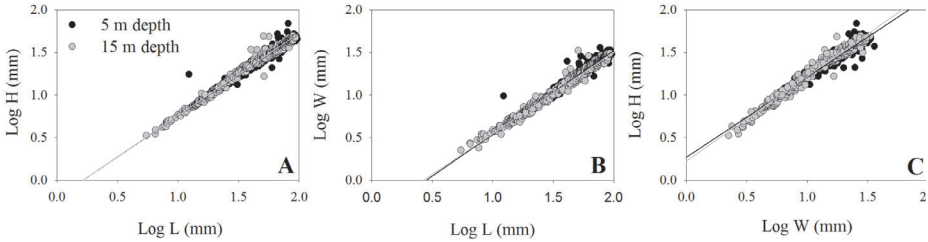

Figure 7: Relationship between log transformed (A) length to height (L vs. H^{-1}), (B) length to width (L vs. W^{-1}), and (C) height to width (W vs. H^{-1}) of the valves of Mytilus galloprovincialis Lmk. *cultured in both culture depth levels (5m and 15m depths; black and grey points, respectively) in the experimental site during the analyzed period (14 months).*

Based on ANCOVA analysis (Table 4), the factor of culture depth significantly affected the linear relation between log transformed shell length (mm) and log transformed shell height (mm). Higher values were found in mussels cultured at the lower depth. No ANCOVA analysis was performed to compare the linear relation between log transformed shell width (mm) and log transformed shell length (mm), neither to compare log transformed shell height (mm) and log transformed shell width (mm). This is due to the fact that significant interaction between the factor (culture depth) and either the covariable log transformed shell length (mm) or the covariable log transformed shell width (mm) was observed. Hence, no direct relation could be assessed between the change in shell height (mm) and the change in shell length (mm), nor between the change in shell height (mm) and the change in shell width (mm) in mussels cultured at both depths.

Table 4 *Mytilus galloprovincialis* Lmk. Culture depth effects on shell width (mm) to shell length (mm); shell height (mm) to shell length (mm); and shell height (mm) to shell width (mm) allometric relations analyses. Culture depth effects were assessed by comparing the allometric exponent b data using ANCOVA (Sokal and Rohlf, 198), with α set at0.05.

Allometric relation	*Source of variation*	*df*	*ANCOVA F*	*sig*	R^2
Log H / Log L	*Log Length (covariable)*	1	36385.798	0.000	
	Culture depth (factor)	1	7.714	0.006	0.968
	Error	1197			

The seasonal allometric relationships between shell length (mm) and shell height (mm), shell length and shell width (mm), or between shell width (mm) and shell height (mm) of mussels cultured at both culture depths are shown in Table 5 and Figure 8.

During fall, based on seasonal ANCOVA analysis (Table 6), the factor of culture depth significantly affected all the linear relations between log transformed shell morphometrics. Higher values were observed in mussels cultured at the lower depth. In winter, the factor of culture depth did not affect the linear relation between log transformed shell length (mm) and log transformed shell height (mm), nor the linear relation between log transformed shell width (mm) and log transformed shell length (mm). However, it did affect the linear relation between log transformed shell height (mm) and log transformed shell width (mm). Higher values were again observed in mussels cultured at the lower depth. Finally, in summer, no ANCOVA analysis was performed to compare the linear relation between log transformed shell morphometrics. This is due to the fact that significant interaction between the factor (culture depth) and either the covariable log transformed shell length (mm) or the covariable log transformed shell width (mm) was observed. Hence, no direct relation between the change in one of the shell morphometric measurements (mm) and the change in another shell morphometric measurement (mm) of mussels cultured at both culture depths could be assessed.

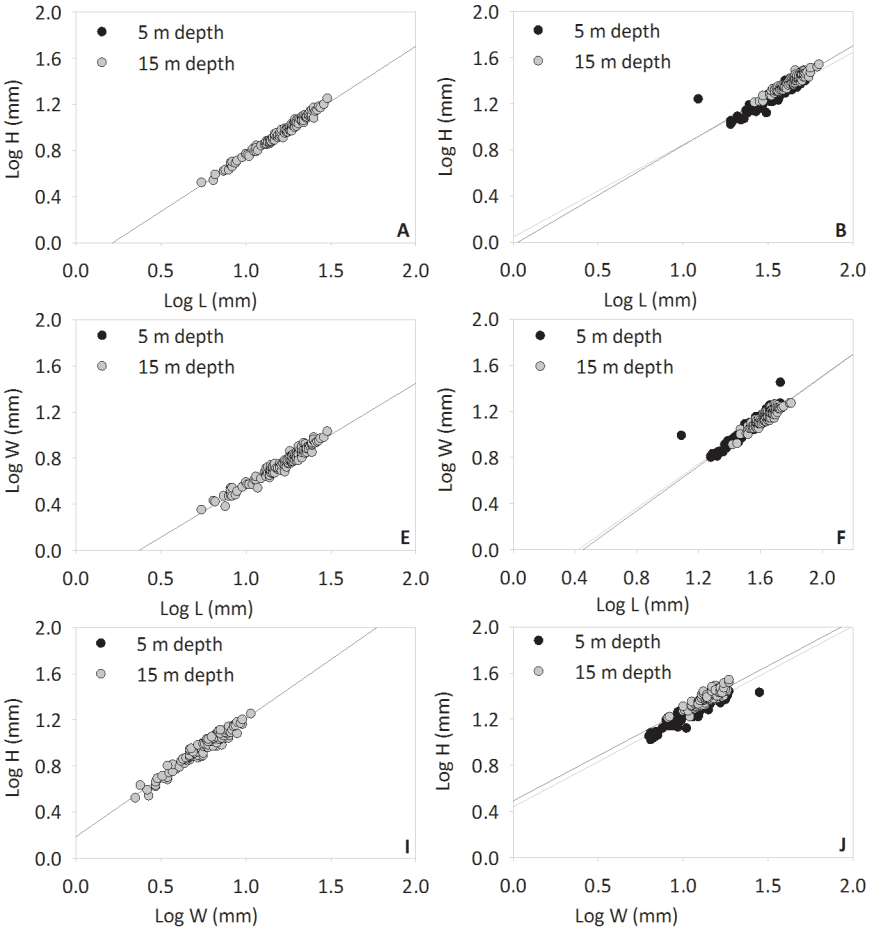

Figure 8 *Seasonal relationship between log transformed (A) length to height (L vs. H^{-1}), (B) length- width (L vs. W^{-1}), and (C) height to width (W vs. H^{-1}) of the valves of Mytilus galloprovincialis* Lmk. *cultured in both culture depth levels (5m and 15m depths; black and grey points, respectively) in the experimental site.*

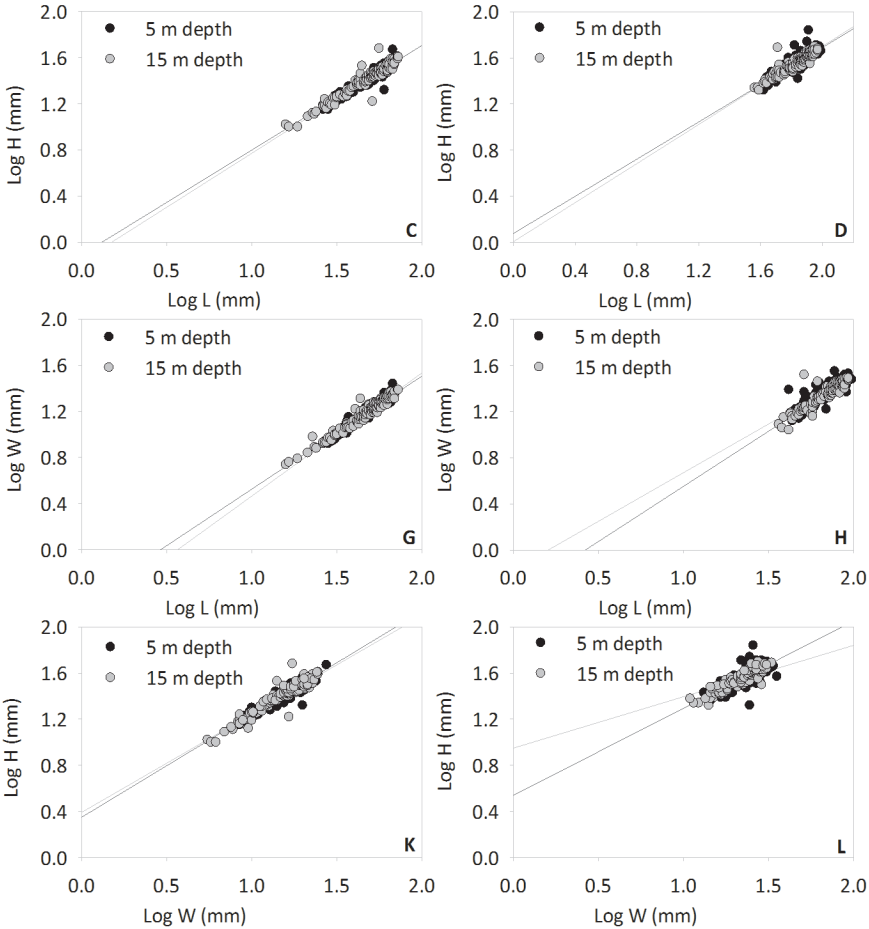
5 m depth
15 m depth
Log H (mm)
Log L (mm)
C
D
Log W (mm)
G
H
K
L
0.0
0.4
0.5
0.8
1.0
1.2
1.5
1.6
2.0

Table 5 *Mytilus galloprovincialis* Lmk. Seasonal parameter of shell shape (length (L), width (W) and height (H)) regression equation used to relate shell width (mm) or shell height (mm) to shell length (mm), and hell height (mm) to shell width (mm) of mussels from different culture depth (Upper depth vs. Lower depth) and sampling time. Equation fitted to log10 X = log10 a + b log10 Y. where a intercept and b slope, and X and Y are shell length measurements. Error of correlation coefficient (r), number of specimens (n) used to derive equation are also given. Culture depth effects were assessed by comparing the allometric exponent b data using ANCOVA (Sokal and Rohlf, 198), with α set at 0.05.

Sampling date	Alometric relation	Depth		B	SE	t	sig	95% CI Upper	95% CI Lower	R^2	N
13-Jun	H/L	5m & 15m	Intercept	-0.206	0.012	-17.215	0.000	-0.230	-0.183	0.985	150
			Slope	0.955	0.010	97.740	0.000	0.936	0.974		
	W/L	5m & 15m	Intercept	-0.329	0.021	-15.641	0.000	-0.371	-0.287	0.948	150
Time Zero			Slope	0.888	0.017	51.826	0.000	0.854	0.922		
	H/W	5m & 15m	Intercept	0.184	0.015	12.037	0.000	0.154	0.214	0.947	150
			Slope	1.026	0.020	51.348	0.000	0.987	1.066		
13-Oct	H/L	5m	Intercept	0.044	0.049	0.904	0.367	-0.053	0.141	0.810	150
			Slope	0.801	0.032	25.115	0.000	0.738	0.864		
		5m	Intercept	-0.019	0.039	-0.484	0.629	-0.096	0.058	0.899	150
			Slope	0.861	0.024	36.214	0.000	0.814	0.908		
	W/L	5m	Intercept	-0.408	0.053	-7.676	0.000	-0.513	-0.303	0.839	150
			Slope	0.959	0.035	27.720	0.000	0.891	1.027		
		15m	Intercept	-0.439	0.051	-8.617	0.000	-0.540	-0.339	0.868	150
			Slope	0.971	0.031	31.246	0.000	0.910	1.033		
	H/W	5m	Intercept	0.445	0.030	14.899	0.000	0.386	0.504	0.839	150
			Slope	0.779	0.028	27.814	0.000	0.724	0.834		
		15m	Intercept	0.494	0.037	13.361	0.000	0.421	0.567	0.800	150
			Slope	0.779	0.032	24.353	0.000	0.716	0.843		
14-Mar	H/L	5m	Intercept	-0.084	0.046	-1.832	0.069	-0.175	0.007	0.877	149
			Slope	0.889	0.027	32.495	0.000	0.835	0.943		
		15m	Intercept	-0.094	0.039	-2.443	0.016	-0.171	-0.018	0.912	149
			Slope	0.899	0.023	39.123	0.000	0.853	0.944		
	W/L	5m	Intercept	-0.500	0.047	-10.612	0.000	-0.593	-0.407	0.897	149
			Slope	1.008	0.028	35.951	0.000	0.952	1.063		
		15m	Intercept	-0.444	0.032	-14.041	0.000	-0.506	-0.381	0.948	149
			Slope	0.973	0.019	51.800	0.000	0.936	1.010		
	H/W	5m	Intercept	0.388	0.025	15.632	0.000	0.339	0.437	0.920	149
			Slope	0.855	0.021	41.213	0.000	0.814	0.896		
		15m	Intercept	0.352	0.029	11.990	0.000	0.294	0.410	0.899	149
			Slope	0.893	0.025	36.395	0.000	0.844	0.941		
14-Aug	H/L	5m	Intercept	-0.002	0.070	-0.032	0.975	-0.140	0.136	0.772	150
			Slope	0.852	0.038	22.401	0.000	0.777	0.927		
		15m	Intercept	0.607	0.072	8.436	0.000	0.465	0.750	0.542	146
			Slope	0.520	0.039	13.223	0.000	0.442	0.597		
	W/L	5m	Intercept	-0.178	0.146	-1.220	0.224	-0.466	0.110	0.434	150
			Slope	0.846	0.079	10.658	0.000	0.689	1.003		
		15m	Intercept	0.452	0.103	4.381	0.000	0.248	0.656	0.342	146
			Slope	0.494	0.056	8.771	0.000	0.382	0.605		
	H/W	5m	Intercept	0.948	0.069	13.724	0.000	0.811	1.084	0.349	150
			Slope	0.446	0.050	8.913	0.000	0.347	0.545		
		15m	Intercept	0.765	0.067	11.456	0.000	0.633	0.897	0.489	146
			Slope	0.585	0.049	11.902	0.000	0.488	0.682		

Table 6 *Mytilus galloprovincialis* Lmk. Seasonal culture depth effects on shell width (mm) to shell length (mm); shell height (mm) to shell length (mm); and shell height (mm) to shell width (mm) allometric relations analyses. Regression equation exponent b (slope) were compare using ANCOVA (Sokal and Rohlf, 198), with α set at 0.05.

	Allometric relation	Source of variation	ANCOVA df	F	sig	R^2
13-Oct	Log H / Log L	Log Length (covariable)	1	1521.569219	0.000	0.898
		Culture depth (factor)	1	60.4723939	0.000	
		Error	297			
	Log W / Log L	Log Length (covariable)	1	1650.324898	0.000	0.878
		Culture depth (factor)	1	5.82526927	0.016	
		Error	297			
	Log H / Log W /	Log Length (covariable)	1	1421.715119	0.000	0.893
		Culture depth (factor)	1	141.3966264	0.000	
		Error	297			
14-Mar	Log H / Log W /	Log Length (covariable)	1	2922.821885	0.000	0.907
		Culture depth (factor)	1	4.032370138	0.046	
		Error	297			

Biochemical composition and fatty acids

The observed values on biochemical composition are shown in Figure 9. No statistical differences were found for any of the biochemical parameters analyzed between the two culture depth levels (Two-way ANOVA, $p>0.05$). Therefore, an integrated biochemical analysis was developed to characterize the composition of the mussels (Table 7.). In general terms, the following pattern was observed: (i) mussel inorganic content decreased in fall (October 2013); (ii) mussel lipid and protein decreased in winter (March 2014); and (iii) a rapid increase in lipid was observed in summer (August 2014). At the two culture depth levels, the maximum level of glycogen was found in both winter (March 2014), and summer (August 2014) ($42.46 \pm 34.47 mg \cdot g^{-1}$ and $49.76 \pm 18.19 mg \cdot g^{-1}$, respectively).

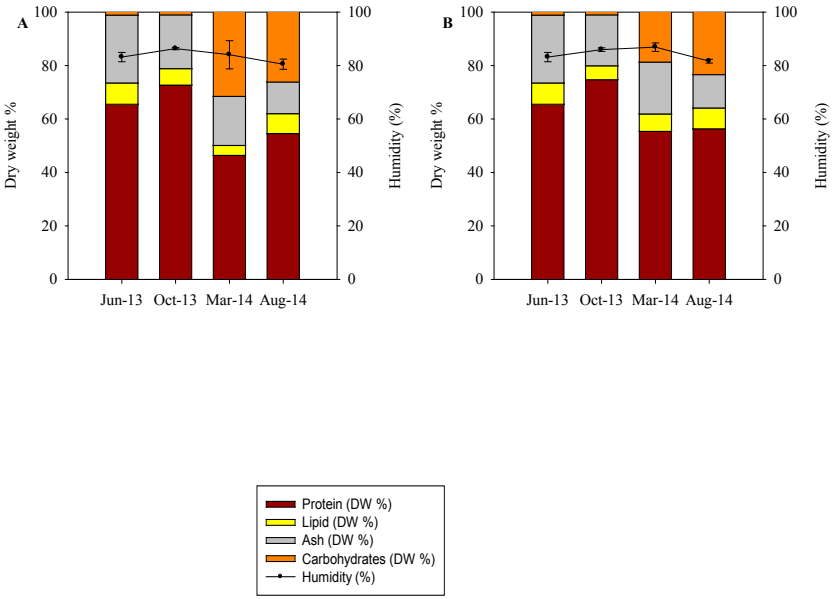

Figure 9 *Biochemical composition based on inorganic (grey), lipid (yellow), protein (brown), carbohydrates (orange) and water (black dotted line) from Mytilus galloprovincialis* Lmk. *mussel pools analyzed at the each sampling time (June and October 2013; March and August 2014) cultivated in (A) 5 m and (B) 15 m culture depth levels.*

Table 7 Average mean biochemical composition (mean ± SD) of mussel (*Mytilus galloprovincialis* Lmk.) from both culture depth levels (5 m and 15 m depths) by sampling time.

Biochemical composition		*Jun-13* TIME ZERO	*Oct-13* Fall	*Mar-14* Winter	*Aug-14* Summer
Dry Weight %	Protein (%)	65.51 ± 2.61	73.71 ± 1.56	50.85 ± 7.75	55.44 ± 4.21
	Lipid (%)	7.98 ± 0.87	5.66 ± 1.00	5.13 ± 1.90	7.61 ± 1.99
	Ash (%)	25.38 ± 3.00	19.59 ± 1.20	18.90 ± 4.39	12.18 ± 1.77
	Carbohydrates (%)	1.13 ± 0.13	1.05 ± 0.22	25.11 ± 12.58	24.77 ± 6.71
Humidity %		83.17 ± 1.56	86.15 ± 0.53	85.46 ± 3.81	81.06 ± 1.43

In total, 34 fatty acids were identified. The observed mean values of fatty acids and their pair wise comparisons between the two culture depth levels at each sampling time are shown separately in Table 8. Most of fatty acids underwent statistical significant changes during the study period.

SAFAs, MUFAs, and PUFAs accounted for the 25, 20, and 55% of the total fatty acid composition found, respectively. These fatty acids varied significantly with time (two-way ANOVA, $p<0.05$). However, significant differences were not found (ANOVA, $p>0.05$) among these fatty acids when the two culture depth levels were compared. SAFAs and MUFAs decreased in winter, while PUFAs decreased in summer.

In the case of SAFAs, palmitic acid (16:0) was clearly predominant, followed by stearic acid (18:0), and by myristic acid (14:0). Palmitoleic acid (16:1 ω7) was the most abundant MUFA in mussel seed, whereas eicosenoic acid (20:1 ω7) was the most abundant MUFA afterwards. The second major FA was oleic acid (18:1 ω9) in fall and summer, and palmitoleic acid (16:1 ω7) in winter.

With regarding to PUFAs, eicosapentaenoic acid (EPA) (20:5 ω3) was the most abundant in mussel seed, whereas docohexaenoic acid (DHA) (22:6 ω3) was the most important fatty acid as mussels grew. In all sampling, the second major fatty acid was hexadecatetraenoic acid

(16:4 ω3).

There was a seasonal and inverse fluctuation of EPA and DHA. The DHA/EPA ratio showed statistical significant differences between the two culture depth levels in autumn (October 2013) and in winter (March 2014), but no differences were found at the end of the study period (August 2014).

A high level of the ω3/ω6 ratio was observed. Low levels of ω6 (from 5.5 ± 0.8% in the shallower culture to 5.6 ± 0.8% in the deeper culture) and high levels of ω3 fatty acids (ranging from 46.1 ± 5.5% in the shallower culture to 45.6 ± 4.8% in the deeper one) were found in all samples. The ω3/ω6 ratio was above 5 during all samplings, and the maximum levels were found in summer (August 2014). Significant higher ratio were found in winter (March 2014) and in summer (August 2014) in the shallower culture.

The percentage of some fatty acids (14:0, 14:1, 16:1ω7, 20:1ω7, MUFA, 20:5ω3 and NMID) decreased as mussels grew at the culture site. On the other hand, some fatty acids (17:0, 18:4 ω3, and terrestrial fatty acids (18:2 ω6 + 18:3 ω3)) increased their content percentage during the present study. Some fatty acids (i.e., arachidic acid (20:0) and eicosatetraenoic (20:4 ω3)) were stable over the whole study period. In general terms, higher proportions of 22:2 NMIDa, 22:5 ω6 fatty acids,

Table 8 Fatty acid composition (% of total fatty acids) of mussels (*Mytilus galloprovincialis* Lmk.) from each sampling time at both culture depth levels tested. * Statistical significance was set up at ($p < 0.05$) (pair-wise comparison). n.d. (Not detected).

	Initial Seed		*5 m depth*			*15 m depth*	
Fatty acids	*June 2013*	*October 2013*	*March 2014*	*August 2014*	*October 2013*	*March 2014*	*August 2014*
14:0	4.02 ± 0.48	2.9 ± 0.07	1.38 ± 0.43	2.74 ± 0.34	2.71 ± 0.21	1.56 ± 0.07	2.72 ± 0.45
15:0	0.39 ± 0.04	0.73 ± 0.04	0.47 ± 0.1	0.75 ± 0.06	0.69 ± 0.01	0.61 ± 0.06	0.79 ± 0.12
16:0	14.43 ± 1.27	16.82 ± 1.12	13.22 ± 3.56	17.96 ± 0.33	15.98 ± 0.23	15.05 ± 1.61	18.6 ± 0.44*
17:0	1.95 ± 0.27	2.54 ± 0.26	2.75 ± 0.67	2.09 ± 0.21	2.59 ± 0.03	2.82 ± 0.43	2.08 ± 0.07
18:0	4.26 ± 0.11	3.68 ± 0.13	4.09 ± 0.43	3.97 ± 0.36	3.55 ± 0.01	4.31 ± 0.53	3.92 ± 0.29
20:0	0.03 ± 0.04	0.03 ± 0.05	*n.d.*	0.05 ± 0.04	*n.d.*	0.09 ± 0.08	0.05 ± 0.05
22:0	0.06 ± 0.08	*n.d.*	*n.d.*	0.03 ± 0.02	*n.d.*	*n.d.*	0.03 ±0.03
ΣSFA	25.13	26.7	21.9	27.58	25.53	24.45	28.18
14:1	1.92 ± 0.16	1.4 ± 0.09	1.53 ± 0.36	0.57 ± 0.07	1.63 ± 0.02*	1.27 ± 0.23	0.51 ± 0.03
16:1ω7	8.93 ± 0.94	1.88 ± 0.03	1.63 ± 0.39	2.25 ± 0.48	1.94 ± 0.1	1.72 ± 0.2	2.33 ± 0.32
16:1ω5	0.27 ± 0.01	0.09 ± 0.15	0.17 ± 0.15	0.28 ± 0.05	0.16 ± 0.14	0.13 ± 0.12	0.29 ± 0.02
17:1	0.56 ± 0.23	0.62 ± 0.12	0.92 ± 0.45	0.33 ± 0.1	0.74 ± 0.11	0.77 ± 0.26	0.41 ± 0.12
18:1ω9	1.02 ± 0.01	2.71 ± 0.01*	1.51 ± 0.51	2.52 ± 0.15	2.45 ± 0.02	1.51 ± 0.24	2.5 ± 0.19
18:1ω7	3.4 ± 0.41	1.09 ± 0.06	1.17 ± 0.36	1.27 ± 0.05	1.07 ± 0.05	1.34 ± 0.14	1.32 ± 0.05
20:1ω11	1.78 ± 0.14	1.58 ± 0.08	1.11 ± 0.17	1.5 ± 0.1	1.66 ± 0.05	1.13 ± 0.11	1.51 ± 0.06
20:1ω9	2.09 ± 0.18	3.35 ± 0.33*	3.24 ± 0.44	3.12 ± 0.35	2.75 ± 0.02	3.81 ± 0.34	3.17 ± 0.12
20:1ω7	1.91 ± 0.17	0.29 ± 0.01	0.5 ± 0.06	0.49 ± 0.03	0.29 ± 0	0.57 ± 0.03	0.55 ± 0.03
22:1	*n.d.*	*n.d.*	*n.d.*	0.05 ± 0.04	*n.d.*	*n.d.*	0.04 ± 0.04
ΣMUFA	21.88	13	11.79	12.36	12.71	12.26	12.63

Fatty acids	Initial Seed June 2013	5 m depth October 2013	5 m depth March 2014	5 m depth August 2014	15 m depth October 2013	15 m depth March 2014	15 m depth August 2014
16:4 ω3	11.58 ± 4.34	12.08 ± 2.59	16.56 ± 5.87	8.65 ± 1.33	13.71 ± 0.03	12.72 ± 2.35	7.38 ± 0.78
18:2 ω6	0.83 ± 0.02	2.28 ± 0.05*	1.54 ±0.19	2.25 ± 0.15	2.01 ± 0.05	1.49 ± 0.04	2.27 ± 0.15
18:3 ω3	0.32 ± 0.38	1.69 ± 0.03	1.53 ± 0.46	1.99 ± 0.36	1.84 ± 0.06 *	1.52 ± 0.19	1.89 ± 0.21
18:4 ω3	0.7 ± 0.03	2.27 ± 0.1	2.75 ± 0.89	3.74 ± 1.09	2.92 ± 0.12 *	2.73 ± 0.25	3.64 ± 0.64
20:2 α	2.3 ± 0.18	4.57 ± 0.16	4.73 ± 1.01	3.24 ± 0.19	4.46 ± 0.14	4.54 ± 0.52	2.96 ± 0.05
20:2 β	1.71 ± 0.1	0.35 ± 0.0*	0.75 ± 0.19	0.26 ± 0.04	0.45 ± 0.01 *	0.68 ± 0.09	0.26 ± 0.03
20:2 ω6	0.37 ± 0.02	0.58 ± 0.01*	0.71 ± 0.15	0.72 ± 0.1	0.53 ± 0.01*	0.81 ± 0.07	0.72 ± 0.05
20:4 ω6	2.55 ± 0.26	2.21 ± 0.19	2.11 ± 0.12	1.77 ± 0.45	1.97 ± 0.02	2.43 ± 0.27	1.96 ± 0.2
20:4 ω3	0.28 ± 0.1	0.24 ± 0.12*	0.3 ± 0.07	0.29 ± 0.12	0.44 ± 0.03*	0.25 ± 0.04	0.29 ± 0.06
20:5 ω3	16.32 ± 1.58	6.01 ± 0.16*	8.77 ± 0.92	8.82 ± 0.16	6.35 ± 0.11*	8.64 ± 0.3	8.86 ± 0.23
22:2 NMIDa	0.23 ± 0.1	1.12 ± 0.01*	0.86 ± 0.13	0.86 ± 0.06*	1 ± 0.02 *	0.9 ± 0.05	0.71 ± 0.04
22:2 NMIDb	6.42 ± 0.24	1.88 ± 0.06*	3.09 ± 0.67	1.44 ± 0.09	2.15 ± 0.05*	3.09 ± 0.21	1.32 ± 0.04
21:5 ω3	1.08 ± 0.06	1.41 ± 0.02	2.27 ± 0.15	1.34 ± 0.08*	1.4 ± 0.03	2.29 ± 0.06	1.21 ± 0.02
22:4 ω6	0.48 ± 0.02	0.27 ± 0.03*	0.31 ± 0.03*	0.3 ± 0.1	0.21 ± 0.01	0.31 ± 0.03*	0.26 ± 0.01
22:5 ω6	0.21 ± 0.04	1.09 ± 0.04	0.83 ±0.15	0.76 ± 0.1	1.4 ± 0.76	0.85 ± 0.09	0.83 ± 0.01
22:5 ω3	2.09 ± 0.29	0.87 ± 0.02	0.97 ± 0.03	1.13 ± 0.03	0.86 ± 0.03	0.99 ± 0.03	1.09 ± 0.05
22:6 ω3	5.54 ± 0.26	21.37 ± 1.68	18.24 ± 1.58	22.49 ± 1.45	20.06 ± 0.4	19.03 ± 0.66	23.53 ± 1.08
ΣPUFA	53.00	60.29	66.31	60.06	61.77	63.30	59.19
NMID	6.65	3.01	3.95	2.30*	3.15	4.00	2.03
Terrestrial	1.14	3.97	3.07	4.25	3.85	3.01	4.16
ω3 /ω6	5.92	5.26	6.34*	6.85*	5.54	6.03	6.70
DHA /EPA	0.34	3.55*	2.08	2.55	3.16	2.20*	2.66

and DHA/EPA ratio were found in fall (October 2013), whereas the contents of 17:1, 16:4 ω3, 21:5 ω3, 22:4 ω6 and PUFA increased in winter (March 2014). Finally, the terrestrial origin fatty acids (18:1 ω9 and 18:2 ω6) were found to increase in fall and summer.

Table 9. shows the statistics summary from the multiple mean comparisons, comparing average contents of selected fatty acids used as trophic markers. PUFA was the major FA group. SFA level was quite stable from seed source and during the study period, the MUFA level decreased during growth in the site, but was frequently maintained quite stable. In this study, the sum of 18:1 ω7 and odd-branched FAs (bacterial contribution to the diet) were moderate and constant. Low ratio 18:1ω7/18:1ω9 (phytoplankton dietary input · carnivorous dietary input^{-1}). On the other hand, a moderate level of 20:1 ω9 FA was found. A high level of 16:1ω7/16:0 (diatom · dinoflagellate-based diets^{-1}) was also found.

Table 9 Quantification of main fatty acids trophic markers by culture depth and sampling time. No significant differences ($p<0.05$) were found

	Initial Seed	*5 m depth*			*15 m depth*		
Fatty acids	*June 2013*	*October 2013*	*March 2014*	*August 2014*	*October 2013*	*March 2014*	*August 2014*
PUFA / SAFA	2.11	2.26	3.03	2.18	2.42	2.59	2.10
PUFA / MUFA	2.42	4.64	5.62	4.86	4.86	5.16	4.69
16:1 ω7 / 16:0	0.87	0.49	0.54	0.45	0.50	0.50	0.45
16:1 ω7+18:1 ω7	12.33	2.97	2.80	3.52	3.01	3.06	3.65
18:1 ω7 / 18:1 ω9	3.33	0.40	0.77	0.50	0.44	0.89	0.53
15:0+ 17:0+ 18:1ω7	5.74	4.36	4.39	4.11	4.35	4.77	4.19

DISCUSSION

Environmental conditions

Growth in marine bivalves is principally affected by seawater temperature, food availability, and salinity (Page and Hubbard, 1987; Rodhouse *et al.,* 1984; Seed, 1976; Widdows *et al.,* 1979). In this study, mussels (*M. galloprovincialis* Lmk.) cultured in the open ocean grew faster from spring to autumn and slower in winter. This observation is similar to other mussel growth studies reported from temperate regions (Kautsky, 1982; Stirling and Okumuş, 1995), where the growth was governed by the same type of interactions. In spring, seawater temperature in the southeastern corner of the Bay of Biscay warms up to 18ºC and 16ºC between 5m and 15m depth levels, respectively; so, the thermocline and stratification conditions seem to occur near the surface at that season. Similarly, in summer the same difference of 2ºC was observed between the two culture depths. Conversely, this trend changes significantly during the winter season, where a thermal homogenization with values of 12ºC covering the full range of the water column over the continental shelf of the region (Collins and Borja, 2004). Since the optimum thermal range for this mussel species physiology is generally reported as 10-20ºC (Bayne *et al.,* 1976), it can be accepted that not only seawater temperatures but also the local mussel species would fulfill the

biological conditions for a suitable growth and survival in the region. During the present study, mussels showed a marketable minimum size of 50-60mm within 9 months of culture and a survival of 90 and 35% between the shallow and deeper culture depth levels, respectively. The values are consistent with the findings reported by Figueras (1990) from the nearest regions to the Bay of Biscay (i.e., Galicia) where mussel raft farming represents a matured industry.

Mean sea current speed did not differ between the selected culture depth levels of the present study, it is noticed, however, that these are significantly higher than the values reported from previous studies carried out in coastal sheltered areas (Camacho *et al.,* 2014). Similarly, highly significant wave height scenarios were also found (with peaks of 7.96 m) to affect mussel growth performance and the afore mentioned winter thermal homogenization during the fall-winter period. Only similar conditions reporting mussel growth from open ocean studies are those of Buck and Buchholz (2004) and Langan (2000), from the German Bight and the Gulf of Maine, respectively. The observed high variability of mussel density within the present study may indicate that not only waves and currents (Witman and Suchanek, 1984; Price, 1982; Brenner and Buck, 2010) but also some other factors (e.g. natural oversets, natural predators, management errors, biofouling, etc.), could

have affected on the seasonal or eventual loss of individuals from ropes. The increase in density at shallower depth was likely due to natural overset, this might indicate that both coastal spawning and settlement of new recruits in the region could mostly occur during the spring and fall seasons, respectively. The self-thinning and rapid dislodgment of mussel individuals when cotton rayon mesh is decomposed could also affect on the mussel density, as previously reported by Fréchette *et al.* (1996) and Fuentes *et al.* (2009). Nevertheless, it is accepted that the ecological approach of the present study provided no attempt to separate the natural set from the original seed. In the context of a commercial farm, all those factors could mask the trends on the expected densities by rope. Therefore a proper management operation, aiming to minimize the negative effects of those factors and their subsequent mistakes, should always be prioritized in open ocean scenarios.

Regarding the phytoplankton biomass, the values of chlorophyll-a measured during the study period were within the range previously reported for this variable in the photic layer of the Basque continental shelf (Revilla *et al.,* 2009, 2010). In this area, similarly to other temperate areas located at mid-latitude, the sea surface temperature differentiates the period of vertical mixing from the stratification period (Valencia *et al.,* 2004; Fontán *et al.,* 2008). These physical processes determine

both, the nutrient concentrations and the retention of phytoplankton biomass in the surface layers of the water column. A combination of cooling, turbulence and downwelling generates the winter mixed layer. The mixing of the water column, together with the frequent rainfall events and freshwater discharges cause a maximum in nitrate and phosphate concentrations during winter. In early spring, a reduction in turbulence allows some degree of stability. Under these conditions, the surface waters receive a high percentage of the total heat flux from the atmosphere and the process is self-enhancing. If the stability is high enough, stratification of waters continues throughout the remainder of spring, all of the summer and the early autumn. The stratification period is characterized by low concentration of nutrients in the surface layers, which limits phytoplankton growth. Under these conditions a subsurface peak of chlorophyll-a can be found around the thermocline (Valencia and Franco, 2004). Storm events, like summer gales produce turbulent mixing and deepen the thermocline. The depth of the thermocline and the associated layers depends also upon the balance between upwelling and downwelling. If downwelling prevails the thermocline moves deeper down. Conversely, if upwelling prevails the thermocline becomes shallower and more distinct. However, it rarely rises up and breaks into the surface layers (Valencia *et al.,* 2004). In the Basque offshore

waters the location of the chlorophyll subsurface maximum seems to be progressively deeper in the water column during the last three decades, being recently placed, in average, at 30 m (Revilla *et al.,* 2010). This trend could have occurred in response to stronger downwelling and reduced cloudiness, coupled with variations in climatic conditions (ICES, 2011). Therefore, in summer, the mussels cultured at the upper level were probably not able to exploit the subsurface chlorophyll peak, being only slightly higher the probability for taking advantage of this food source at the lower level.

In the SE Bay of Biscay, the Chl-a shows a bimodal cycle in surface waters, with maxima that rarely exceed 3 $\mu g \cdot l^{-1}$ around early-spring and mid-autumn, the transition phases between conditions of stratification or mixing. However, during summer, in conditions of thermal stratification, phytoplankton biomass decreased to minimum values ($<0.2\ \mu g \cdot l^{-1}$) in the surface layer (Morán *et al.,* 2012). Here, this cycle was also observed, although probably not all the peaks and minima in the chlorophyll-a could be detected due to the limited frequency of sampling for the high variability of the phytoplankton. In this regard, the short-term variability of the meteorological and hydrographical factors can overlap with the typical seasonal cycle and influence the phytoplankton dynamics in coastal waters. For example, inputs of nutrient-rich

continental waters, caused by short periods of atmospheric instability, may act as modulation factors of the bloom decay, during late spring and summer.

Food availability is generally the single most important environmental variable regulating mussel growth performance and efficiency when temperature is constant (Griffiths and Griffiths, 1987; Hawkins and Bayne, 1992; Steffani and Branch, 2003). Sea current speed and turbulence will likely be major factors controlling the mussel growth and survival success during the coldest seasons in the region. On the contrary, during the warm seasons, phytoplankton biomass could be a limiting factor. The continental shelves of the western Iberian Peninsula (Portugal and Galicia) are strongly affected by upwelling pulses that generally occur during spring-summer (Álvarez *et al.,* 2008). These natural events are caused by the prevailing winds and the coastline orientation, causing an inflow of deep nutrient-rich waters that boosts phytoplankton productivity (Varela *et al.,* 2005). However, the intensity and frequency of upwelling pulses decrease eastward along the southern Bay of Biscay (Mason *et al.,* 2005; Lavín *et al.,* 2006). Therefore, the influence that these processes could have on the Basque shelf is considered negligible in terms of phytoplankton biomass increments (Revilla *et al.,* 2009). The utilization of non-phytoplankton food sources (such as

the non-living part of the POM or microzooplankton) by mussels in the study area when this resource concentration is seasonally low (i.e., summer) can be hypothesized.

Relative growth and condition

Growth rate was measured in terms of length and weight. Body shape does not always change uniformly with an increase in length, but it depends on the allometry of growth which can be regulated by multiple factors (i.e., genetics, crowding, trophic conditions, water depth, wave impact or presence of predators, among others) (Akester and Martel, 2000; Brown *et al.,* 1976; Eager, 1978; Fox and Coe, 1943; Reimer *et al.,* 1995; Seed, 1978; Steffani and Branch, 2003).

At the end of the study, the selected culture depth levels (5m and 15m depths) produced almost similar mussels from a growth and condition perspective. Indeed, an important settlement of natural seed was also noticed during the same period and depth level; this was not noticeable in mussels from the deeper culture. This finding might represent a simple seasonal morphological adaptation by mussels to allow individuals to grow better for a longer time period prior to being physically restrained by the new neighboring individuals and encountering less restriction in valve opening than those that are dorso-ventrally compressed (Fréchette and Despland, 1999; Lauzon-Guay *et al.,* 2005). Thereafter, the more

extreme winter hydrodynamics might have contributed to dislodgement of larger individuals and reduction of seasonal densities on the ropes, but at the same time masking any difference in shell morphology between the selected culture depth levels. The mussel shells of the present study were characteristically round, wide and great in thickness. Although these observations were not validated through specific comparisons, they are consistent with the findings from previous studies developed with mussels from wave beaten shores (Akester and Martel, 2000, Lewis and Powel, 1961, Seed 1968).

The relationship between shell length and meat weight remained identical, between both sets of culture depth conditions up to the end of the study. Only in the fall (October 2013), did mussel growth of shell weight (SW) and body weight (DMW) display higher rates in the deeper culture zone. These differences could be related to an inherent slower mussel growth of individuals having heavier shells (i.e., an inverse relationship of shell thickness with growth) or to a secondary effect of size-selective mortality in nature (Seed, 1980; Steffani and Branch, 2003).

The results in winter showed that shell growth can increase even in periods when tissue growth is stagnated. That observation supports the findings of Fréchette and Bouget (1985), Borrero and Hilbish (1988),

and Alunno-Bruscia *et al.* (2001) who reported that shell growth is not necessary coupled with tissue growth in bivalves.

The lowest condition indexes were found in fall and in winter. These findings might explain the energetic expenditures of major biological efforts that mussels have to display during those poor environmental seasons to maintenance or produce gametes in the Bay of Biscay. This observation is also corroborated by the high proportion of ash content observed in mussel individuals during the same dates and consistent with other authors (Lucas and Beninger (1985) and Frolov *et al.* (1995)) who also reported high proportions of ash content associated to natural stress response or depleted energy reserves.

The condition index, expressed as allocation of resources to tissue or shell growth, followed a seasonal trend during the whole period of study; such observations have already been extensively reported by several authors (Hickman and Illingworth, 1980; Okumuş and Stirling, 1998; Smaal and Van Stralen, 1990; Thippeswamy and Joseph, 1992). However, Rainer (1992) and Iglesias *et al.* (1996) also reported species and geographical location-specific condition index responses to environmental change. Therefore, the results can be an important baseline information for use to evaluate mussel physiological status by season in the region.

The fluctuations in condition index and meat weight have important implications for cultivation and harvesting strategies. For optimum exploitation the harvesting season should be timed according to the peak period for condition index (Okumuş and Stirling, 1998). Similarly, Mason (1969) stated that DMW condition index of 10 are acceptable for marketing. Since the results of condition index at the present study were generally higher than 10, it can be outlined that mussels cultured at the region will surely be suitable for marketing all year round except the winter season.

Biochemical composition and fatty acids

The proximate composition of mussels cultivated at the experimental site underwent a seasonal biochemical cycle characterized by phases of accumulation and depletion of reserves. The fluctuations of total lipids, glycogen, protein and ash content were found to depend on seasonality but not on culture depth.

High levels of glycogen were observed in the shallower and in the deeper culture zones in winter and summer, respectively. The glycogen peaks in the 5m culture zone are likely related to early gametogenesis whereas another factors like egg reserves or delayed gametogenesis, as reported by Gallardi *et al.,* 2014, could be responsible for glycogen retention in summer in the deeper culture zone. Similarly, lipid content

in mussels steadily increases during summer months; this is consistent with Prato *et al.* (2010) and very likely related to the storage cycle (Gabbot, 1983) of this species in the region of study; albeit reproductive biology was not a focus of the present contribution.

At the end of the study, mussel products from both culture depth levels presented similar proximate composition values than the ones described by Fuentes *et al.* (2009) from mussels (*Mytilus galloprovincialis* Lmk.) cultured at sheltered areas in the SW Bay of Biscay (i.e., Galicia). Fatty acid profile, palmitic (16:0) and gondoic acid (20:1ω9) acids were found to be the predominant SAFAs and MUFAs in the final mussel products. This is consistent with Alkanani *et al.* (2007), Freites *et al.* (2002b) and Orban *et al.* (2002).

Among PUFAs, EPA (20:5 ω3) and DHA (22:6 ω3) were the most prevalent fatty acids with the maximum levels of DHA being found in the summer samples. This is consistent with Abad *et al.* (1995) and with different studies from the Mediterranean (Karakoltsidis *et al.,* 1995), Atlantic (Fernández-Reiriz *et al.,* 1996), and Adriatic sea (Orban *et al.,* 2002) reporting similar fatty acid profiles in the mussel *Mytilus galloprovincialis* Lmk. The average proportion of healthy PUFAs, in the mussels from both culture depths, was quite stable during the whole study period and it amounted to 53-66% of total fatty acids, 21-28% of

SAFA and 11-21% of MUFAs. These proportions are consistent with Alkanani *et al.* (2007), Dridi *et al.* (2007), Frolov *et al.* (1995), Freites *et al.*(2002b) and King *et al.*(1990).

The presence of NMID (22:2) fatty acids was also detected. This is consistent with previous observations on *Mytilus* spp., where not only the presence but also positive correlations with DHA were reported (Alkanani *et al.,* 2007).

Here Chl-a was positively correlated with DHA, terrestrial fatty acids (18:1 ω9 and 18:2 ω6) and SDA (18:4 ω3), whereas it was negatively correlated with NMID. Several authors (Fuentes *et al.,* 2009; Ojea *et al.,* 2004) have reported positive correlations between Chl-a, SFA and organic-detritus rich environment with abundant bacterial loads. Our differences could be related to the environmental conditions at our exposed location where water renewal rate (due to currents and waves) is expected to be higher than at sheltered areas.

Budge *et al.* (2001) suggested that optimal values of the ratio ω3/ω6 PUFA were between 5 and 15 for mussel growth. Herein, mussels showed a stable ω3/ω6 ratio from 5.26 to 6.85 in the shallower culture zone and from 5.54 to 6.70 in the deeper culture zone. These values would support the consistency of the ratio ω3/ω6 in the mussels of the present study; although, they can be considered medium level concentrations

and very likely influenced by the more limited capability of mussels from exposed locations for de novo synthesis of PUFA from the diet.

The contribution of phytoplankton (mostly diatoms and dinoflagelates) to the diet of *M. galloprovincialis* Lmk. in the region is likely moderate. EPA and DHA, fatty acids are known to be synthesized by diatoms and dinoflagellates. This could be supported by the low proportions of 16:1 ω7/16:0 and 20:5 ω3/22:6 ω3 observed during the present study and consistent with other studies from offshore locations (Prato *et al.,* 2010).

Mussel quality characteristics (i.e., condition, composition, glycogen, fatty acids, etc.) as determined by seasonal intervals, reflect the environmental conditions met by the animals only at the specific period considered of their growth. Indeed, as reported by Orban *et al.* (2002) the particular sessile habit of mussels makes their chemical composition strictly dependent on more complex compensating factors such as phytoplankton availability, seasonality, year to year variability of the climatic conditions and/or gametogenic cycle.

Conclusions

The results of the present study constitute the first available ecological data on the culture of mussels (*Mytilus galloprovincialis* Lmk.) at the open ocean scenario of the Bay of Biscay. The findings

would support mollusk aquaculture moving off the coast at this specific location. The high environmental complexity observed at local level (i.e., strong hydrodynamics and low seasonal productivity) would however recommend a precautionary approach. Main growth and biochemical parameters were maximized during summer season. Conversely, the no clear evidences detected for the effect of culture depth on meat quality suggest that a difference of 10 meters depth might not be enough to promote final differences on a mussel product from this type of exposed scenarios. In any case, proper management and operation strategies could always benefit on density, growth and mussel condition at seasonal level. The results of this contribution provide useful information on: (i) mussel local growth performance and biochemistry; and (ii) characterization of the local environmental scenario regulating further developments of mussel industry within the region. This contribution will also benefit local aquaculturist and all of those involved in entrepreneurship related to open ocean aquaculture. Future research efforts should be directed towards (i) the search of efficient mussel production technologies and (ii) a correct definition of carrying capacities, both considering the environmental and socioeconomic restrictions of the region.

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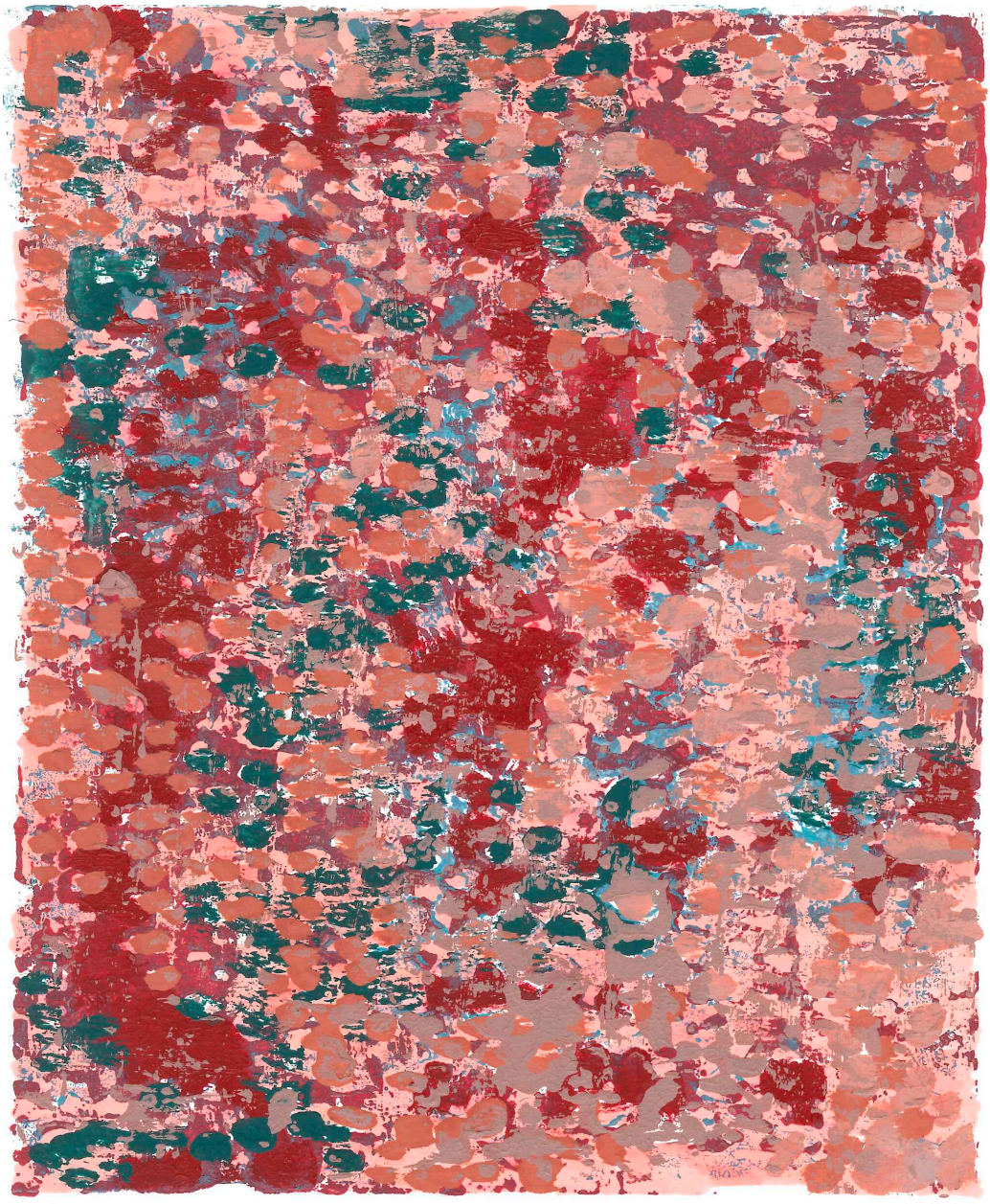

CHAPTER 5

CULTURE PRODUCTION, GROWTH RATE PATTERNS AND PRODUCT QUALITY OF MUSSEL (*Mytilus galloprovincialis* LMK.) CULTURED AT TWO DIFFERENT DEPTHS IN THE OPEN OCEAN OF THE SE BAY OF BISCAY FOR THE COMMERCIAL PRODUCT DEVELOPMENT.

SUMMARY

Mytilus galloprovincialis Lmk. mussels were cultured at two culture depths in a submerged longline system, located in the SE Bay of Biscay, from June 2013 to August 2014. The Von Bertanlanffy Seasonal Growth Function (VBSGF) of the original seeded batch, without newly settled, at each culture depth was assessed. During winter, growth rate stabilizes and mussels grow more slowly. Mussels maintain their reached size during these slow growing months and increases thereafter. In addition, to test both culture depth scenarios for resource management, the production of commercial pieces ("small" and "large" mussels) in quantity (mussel classification), as well as in quality, was assessed. Quality was assessed through condition index, proximate composition and fatty acid composition of mussels harvested in winter and summer. In order to harvest mussels of a commercial size with a CI above 10, the optimum seeding time at the studied area may well be in early fall (September) and in this situation harvesting may well be optimum from the following early fall till the end of the slow growing period. VBSGF output proved that commercial mussels may well be harvested throughout the year in the studied site. Depending on seeding time, culture period to produce mussels of a commercial size will last one year or one-and-a-half years. Depth did not appear to have an effect on quality characteristics in

mussel of both commercial sizes. This work was also the first attempt to identify major food sources of mussels of different sizes, using fatty acid trophic markers (FATM), of *M. galloprovincialis* Lmk. cultured in the SE Bay of Biscay. Regardless of the feeding differences observed between mussel products, both products displayed similar quality characteristics. Hence, harvesting mussels in the studied area is likely to be driven by logistical considerations rather than by quality differences.

keywords: Mussel; *Mytilus galloprovincialis* Lmk.; Open Ocean; Growth; Condition Index; Biochemical Composition; Length Structure

INTRODUCTION

Growth rate and Production

In temperate waters food supply is the most important factor that affects mussel growth, as tissue production relies on food availability and the energy ingested as part of the diet must exceed the cost of metabolic maintenance for growth and reproduction to occur (Hawkins and Bayne, 1992). However, the effect of other parameters such as temperature cannot be ignored (Bayne and Newell, 1983; Page and Hubbard, 1987). Particularly, temperature and growth seem not to be linearly related when the range of temperatures is too wide (Ramón *et al.,* 2007). Caciun (1980) considered a temperature of 25°C as the upper thermal limit for the optimal development, growth, and survival of *Mytilus galloprovincialis* (Lmk). Similarly, Almada-Villela (1982), found that growth rate of *Mytilus edulis L.* declines sharply below 3°C and over 20°C. Hence, growth is slower in colder months. A number of other factors influencing growth include depth, location of culture systems, position of bivalves relative to the water current and culture densities.

Growth can be measured as change in weight or length with time. Growth, perceived as a linear, exponential or asympotic relationship between the animal's size and culture time, can be numerically represented as growth rate (Hopkins, 1992). Shell length measurements

of individuals are the most widely used technique for measuring growth rates of a population (Quayle and Newkrik, 1989). Techniques for measuring this growth rate include measuring the length of individual mussels of a population at a time, successively measuring marked individual mussels over a period of time, as well as counting annual growth rings on mussel shells. These techniques each have their own advantages and disadvantages (Seed, 1976).

Each technique is applied to a mathematical model in order to quantify the average growth rate of the population. Growth is an asymptotic sigmoid curve (Hopkins, 1992). Bayne (1965) was first to explain that *Mytilus* growth is linear during the larval period and asymptotic thereafter. Similarly, Andreu (1958), Aguirre (1979), and Pérez-Camacho and Román (1979) stated growth rate of *Mytilus galloprovincilais (Lmk)* is constant below a shell length of approximately 60mm and then declines as the animal reaches its maximum attainable length (i.e., asymptotic length).

Aquaculturists are not interested in the maximum attainable size of the animal but rather, they are interested in the moment mussels attain commercial sizes. Most of these mathematical models are limited to the adult life stage of the animals, and they do not include all the different growth patterns that a bivalve may display during their lifetime. Despite

these limitations, many studies on *Mytilus galloprovincialis* Lmk. population growth have been done in the SW Bay of Biscay (i.e. Galicia) (Fuentes *et al.,* 1992; Pérez-Camacho *et al.,* 1995; Fernández-Reiriz *et al.,* 1996; Babarro *et al.,* 2003 and Ramón *et al.,* 2007) as well as in the Mediterranean Sea, mostly in Adriatic waters (Hrs-Brenko, 1973; Ceccherelli and Rossi, 1984; Fabi *et al.,* 1989 and Orban *et al.,* 2002). Similarly, this present supplementary study aims to assess the growth rate of the seeded population described in Azpeitia et al. (2016). This was the first offshore mussel production in the SE Bay of Biscay in a new pilot submerged structure, and the study lasted 14 months. The ecology of this first mussel culture was described with the aim of assessing how culture depth affects general growth (seeding and final harvest) and general quality of all the mussels present (harvested) on ropes. The instantaneous growth rate (G) of the final product (14 months) harvested on ropes was measured regardless of the hypothesized natural settlement. However, from a producer's point of view it is well worth assessing the growth rate of the seeded population alone. Settlement may vary annually and thus, producers cannot depend on settlement for predictable production, nor can they control its variability.

Seed (1976) and Kautsky (1982) reported that the estimation of the population growth rate from length-frequency distribution data may

have limitations since *Mytilus* display high extended annual recruitment periods and a high variability of individual growth rate. Nevertheless, some authors (Bayne and Worral, 1980; Page and Hubbard, 1987) assessed growth by measuring the change in length-frequency distribution of recognizable cohorts of a population in a period of time. In these situations, length-frequency distributions could be polymodal with each mode representing an individual age class (cohort) and growth rate could be assessed with the change in position of this mode with time (Bayne, 1965). Moreover, Richardson et al. (1990) measured growth rate from a population of mussels in a newly cleaned offshore platform and found that the length-frequency distributions obtained from that mussel population were polymodal with the modes representing specific individual "relative" age-classes (pseudo-cohorts). This technique of length-frequency analysis is based on the assumption that recruitment is seasonal with one, at most two, peaks pear year. Moreover, this technique assumes that the samples cover the entire range of lengths present in the population. The aim of this technique is to separate complex length-frequency distribution into cohorts. Then an arbitrary age can be assigned to those cohorts (i.e., Bhattacharya, 1967), and hence, analyzing the growth in length of these cohorts over a period of time, seeded population's growth rate can be assessed.

If accurate age of the individuals is not known, but indicative age can be assumed for each estimated mode, from the length-frequency distributions observed; those estimated modes would represent individual "relative" age-classes (pseudo-cohorts). As long as these pseudo-cohorts can be assumed to represent age-classes, a Ford-Walford plot can be used with the mean length of these seasonal modes to estimate the von Bertalanffy growth parameters (King, 1995). With these growth parameters, the mean growth rate of the seeded mussel population can be assessed. The resulting output of von Bertalanffy seasonal growth function (VBSGF) can be used to visualize the season at which growth slows down to a minimum rate, once minimum commercial length is attained. Therefore, this resulting output may well facilitate shellfish harvesting management decisions (e.g., tinning out processes, operational harvesting decision, harvesting optimum time periods for increasing revenues).

The change in individual weight and change in mussel density can also be used to assess the evolution of population biomass and production (Crisp, 1984), which indeed, are of practical importance with respect to the exploitation of shellfish stock. Ash Free dry weight (AFDW) has been considered an accurate method to express this biomass (Palmeri *et al.,* 1994; Boudreau and Dickie, 1992).

Another important aspect of commercial exploitation is the number of marketable mussels at the time of harvest, thus, production. Two commercial mussel products assessed by the shell length of individuals into "small mussel" and "large mussel" can be found in the market (Peteiro *et al.,* 2006; Ramón *et al.,* 2007).

Product quality

The condition index, the gross biochemical composition as well as the fatty acid composition of the two mussel products (i.e., "small mussel" and "large mussel" products) could be compared at seasonal level and spatial level in order to account for any possible differences in product quality (Filgueira *et al.,* 2006; Fuentes *et al.,* 2009; Orban *et al.,* 2002, 2007). Further, data on both mussel product quality and production (number of commercial pieces of "small mussel" and "large mussel" products) could be used for resource and operational management.

The condition index could be used as a measure of physiological status (Seed and Suchanek, 1992; Steffani and Branch, 2003). Similarly, the proximate composition of mussels (which undergoes seasonal cycles) will reflect the gonad development stage, the status of reserves accumulation as well as, to an extent, the food availability in the environment (Orban *et al.,* 2002; Cartier *et al.,* 2004).

Regarding lipids, the seasonal variations in Fatty acids (FA)

compositions of adult bivalves is also closely linked to the reporductive cycle of the animal, climate changes in the habitat, as well as the aforementioned availability and composition of the natural diet (Fernández-Reiriz *et al.,* 1986; Caers *et al.,* 2000). This natural available diet would also vary with temperature, water stratification, and hydrodynamics (Cartier *et al.,* 2004). Certain FA can be used as biomarkers of several weeks prior diet of the mussel (Ezgeta-Balić *et al.,* 2012). These Fatty acid Trophic markers (FATM) may well be use to assess the contribution of phytoplankton classes, bacteria, as well as microzooplankton to the diet of bivalves (Budge *et al.,* 2001; Handå *et al.,* 2012).

Further, the level of other FA such as ω3 fatty acids (i.e., ω3 PUFAs), may also be used to assess the quality of mussel product. In humans the ω3 PUFAs are not effectively produced by their precursor ᾳ-linoleic acid (Burdge and Wootton, 2002; Burdge *et al.,* 2002) and must be obtained from food that is naturally rich in these ω3 FAs, such as marine animals (McLean and Bulling, 2005; Taylor and Savage, 2006). Hence, the effect of culture depth and harvesting season, as well as product size on these omega-3 FA content may well be use in resource management.

The aim of this supplementary study is first to analyze the production and biomass variation of mussels cultured in open ocean scenario in the

SE Bay of Biscay and visualize which had been the real performance of original seed batch cultured at the pilot system. Second to analyze the quality of all the production of mussels of commercial shell length (from original batch plus newly settled mussels) harvested through (i.) somatic and condition indexes (ii.) proximate and fatty acid composition analysis of the two commercial mussel products. The variation in standing stocks, production of commercial pieces in quantity, as well as in quality was assessed in order to test both culture depth scenarios for resource management.

MATERIALS AND METHODS

Experimental site

The Basque coast is located in the SE Bay of Biscay (Fig. 1.A. and Fig. 1.B.). The open ocean experimental site (43° 21.39' N, 2° 26.90' W) is located 2 miles off the coast (Fig. 1.C. and Fig. 1.D.) A detailed description of the pilot culture system can be found in Azpeitia et al. (2016).

Growth

The study was carried out from over fourteen months from June 2013 to August 2014. *Mytilus galloprovincialis* Lmk. juvenile mussels were manually seeded onto 24 growing ropes each of 12m, suspended and distributed (12:12) at two different culture depth ranges from the longline system headlines (5m and 15m depths). The initial mussel mean length and density was 17.40 ± 5.07mm and 681 ± 37 individuals per linear meter respectively. Three mussel ropes from each culture depth (5m and 15m depths) were randomly sampled every 4-5 months, depending on environmental conditions, and transported to the laboratory.

In the laboratory, mussels were detached from ropes. These mussels were then weighed to obtain the total weight on ropes and cleaned ropes were measured to the nearest 0.1cm. Subsequently, a subsample of approximately 15,000g of mussels per individual rope was prepared

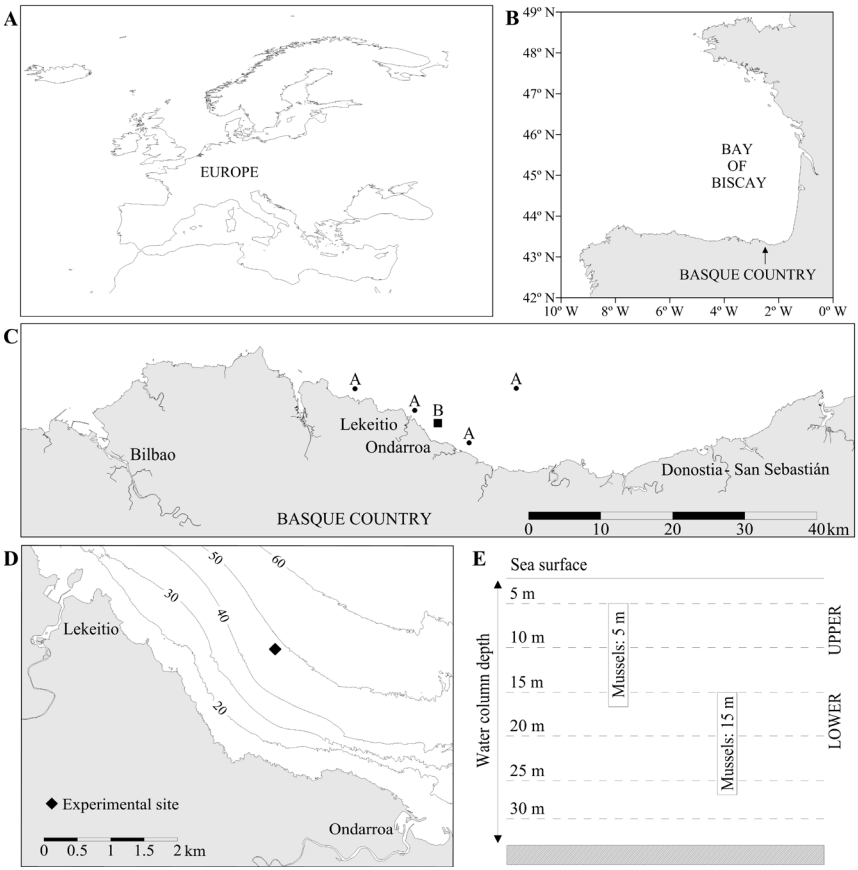

Figure 1 *A) Bay of Biscay location. B) Basque Country coast. C) Map of the study area showing the specific location of the open ocean experimental site (Mendexa: B black square) and the stations for chlorophyll-a data acquisition (A black points). D) Bathymetry map of the experimental site. E) Synthetic scheme of the suspension range of the mussel culture ropes within the water column at the beginning of the experiment.*

for analysis. Individual mussels were carefully detached from each other by cutting the byssal threads and cleaned of encrusting organisms. Individuals were then measured and counted to calculate size frequency distribution. Individuals of the same size class were weighed together to obtain the total weight on ropes of each size class as well as the mean theoretical individual weight per size class. These data were used for the estimation of density (individual's·m-1) and mortality on ropes and for absolute size frequency. Estimates were made based on the 45,000 g of total harvested from 36m of rope (three ropes) per sampling depth and per sampling time.

The measurements of shell length were grouped into 10 mm length-class ranges (constant size classes) and the resulting length-frequency tables were resolved into Gaussian components, using the method of Bhattacharya (1969) with the program FISAT II (Version 1.1.2, FAO-ICLARM Fish Assessment Tools). The mean length of the presumed principal age-class (the original seed batch) from these resolved modal values at each sampling time and culture depth scenario, were fitted to a von Bertalanffy growth function. Based on frequency, size and culture time, mussels with a length size that could not be considered as coming from the original seed batch in fall, winter and summer samplings were considered as a different age-class (newly settled mussels). The von

Bertalanffy growth function used in this study to calculate growth rate was that described by Somers (1988) (VBSGF) and applied accordingly:

$$L(t) = L_{\infty}(1- e)^{- K (t - t0) - S(t) + S(t0)},$$

$$\text{with } S(t) = (c\ K / 2\ \pi) \sin (2\ \pi\ (t - t_s)),$$

$$\text{so } S(t_0) = (c\ K / 2\ \pi) \sin (2\ \pi\ (t_0 - t_s))$$

L_{∞} (mm) is the model asymptote for average length, at which growth is zero. K (year^{-1}) is the measure of the exponential rate to approach the asymptotic length (i.e., growth rate) and C is the parameter that measures the amplitude of the seasonal growth oscillations of the growth curve. When growth increments can be separated into two significantly different groups pertaining to two periods of time of six months, seasonal oscillations is suggested. The parameter t_s (Summer time) is the time between 0 and the start of the convex portion of the first sinusoidal growth oscillation, represented as a fraction of a year ($0 \leq t_s \leq 1$). For visualization, it helps to define $t_s + 0.5 = WP$, which expresses, as a fraction of the year, the period when growth is slowest. WP represents winter point, the time when growth becomes zero during winter or the low growth season.

Commercial sizes at harvest

Two shell length-class range groups were established to compare the quality characteristics of mussels above minimum commercial

length ("small mussels" (50-70mm) and "large mussel" (>70mm)) on spatial (the two different culture depths scenario) and temporal (seasons where those commercial mussel products were harvested) scales. These two mussel products are related to commercial mussel culture phases used in other growth studies (Peteiro *et al.,* 2006; Ramón *et al.,* 2007). No individuals of pre-fattening stage (i.e., <50 mm; named as "mussel below minimum commercial size" by Peteiro *et al.* (2006)) were used in this part of the study. Comparisons were made between "small mussels" (i.e. from thinning-out culture phase; 50-70 mm) and "large mussels" (i.e. from harvest phase; >70 mm) (Peteiro *et al.,* 2006; Ramón *et al.,* 2007).

Condition index

Mussel shell and associated meat were carefully dissected away, separately placed in pre-weighed porcelain trays for drying at 80°C for 24 h and then weighed to the nearest 0.001 g (Walne, 1976). Subsequently, dry mussel meat was individually ashed at 450°C for 4 h in a muffle furnace (Brake *et al.,* 2004), cooled in a desiccator and weighed again to determine the ash-free dry weight content (Lucas and Beninger, 1985).

Biochemical composition

Biochemical condition and fatty acids composition of the same commercial mussel product ("small" and "large" mussel products) harvested during the present study were assessed. 3 replicate samples

of 10-20 pooled mussel individuals from each commercial length-class range available per depth/sampling time were considered for biochemical characterization (including proximate and glycogen analysis) and lipids determination (including total lipids and fatty acids).

The fresh individuals were firstly minced in a food processor (IKA® M 20 universal mill, IKA 1603601, Germany). Thereafter, the samples were analyzed for moisture and ash according to gravimetric methods and total protein determined according to Kjeldahl´s method (AOAC, 1975). Samples were digested in a digestor BUCHI B-435, distilled using a BUCHI B-324 distillation unit and automatic volumetric titration, developed through a Mettler Toledo T- 50 (Mettler Toledo, United States). Finaly, glycogen concentration ($mg \cdot g^{-1}$ mussels' tissue) was calculated based on a mussel glycogen standard (Sigma, Saint Louis, MO) following the methodology reported by Gallardi et al. (2014).

Total lipids were determined based on Blight and Dyer method (Hanson and Olley, 1963). In this way, total lipids from a known weight of tissue was extracted and purified by homogenization in a suitable excess of chloroform/methanol/water (2:2:1.8 v/v/v). Following this, total lipids in chloroform solution were determined by weighting the

eluate after removal of the solvent by rotary evaporation. All lipid extracts were then stored in chloroform at -30ºC to await analysis.

Fatty acids

Fatty acids were determined from the lipid samples. Fatty acid methyl esters (FAME) were separated and quantified using an Agilent 7890 Gas Chromatographer equipped with auto-injector (Agilent Technologies, United States). The gas chromatograph is equipped with a ame-ionization detector (290°C) and a DB-WAS 122-7032 Agilent Technologies capillary column (30m _ 0.25mm I.D., film 0.25µm). Helium was used as a carrier gas and initial oven temperature was 50°C, followed by an increase at a rate of 10°C·min^{-1} to a nal temperature of 240°C for 9min. Individual FAME were identified by comparison of retention times with four commercial fatty acid standards (PUFA 1, PUFA 3, BAME and 37-component) supplied by Supelco (Bellefonte, PA) for area percent normalization. At each sample relative quantities were expressed as weight percent of total fatty acids.

Statistics

The model to fit size frequency distributions in Von Bertanlanffy Seasonal Growth function is available in FiSAT (FAO-ICLARM Stock Assessment tool) software program (Gayanilo *et al.,* 2005). This model is a modification of von Bertanlanffy growth model, which allows for

seasonal oscillations in growth, within each age-group.

These parameters of von Bertalanffy growth function were assessed using ELEFAN I (electronic length-frequency analysis) (Pauly and David, 1981; Gayanilo *et al.,* 1988). The growth increment data was created by the linking of modal mean lengths (resulting in a relative size-at-age data). It is recalled that this growth curves were established by ELEPHAN I without any external input as to the true age-structure of the populations. A forced Gulland and Holt routine was done for preliminary identification of growth oscillations, through an iterative routine which identifies the time of the year that maximizes the differences between the mean of two sets of residuals, covering 6 months each. *t-test* was used in this routine to identify significant differences and a preliminary estimation of C and WP was given.

Subsequently, these C and WP values with ELEFAN I routine were used to identify the seasonal oscillation curve that "best" fit the case-study set of length-frequency data, using the value of R_n as a criterion.

$$Rn\ (10^{ESP/ASP}/10)$$

ASP represents the "Available Sum of Peaks" computed by adding the best values of available peaks and *ESP* represents the "Explained Sum of Peaks" computed by summing all peaks and troughs "hit" by the resolved growth curve (Gayanilo *et al.,* 2005).

In von Bertanlanffy growth function t_0 is included to adjust the equation for the initial size of the organism and is defined as the age at which the organism would have had zero size. Usually, it is replaced by the coordinates of a point through which the curve must pass and whose coordinates consist of a starting sample (SS) and starting length (SL). A mussel size of 20mm shell length, used for seeding (time zero, June), was used as t_0 value.

Data on quality characteristics of mussel products were plotted using Sigmaplot (11.0) statistical and graphical software (Systat software) and statistical analysis was carried out using a statistical package (SPSS 17.0 for Windows®). Data were tested for normality (Shapiro-Wilk test) and variance homogeneity (Leven's test). Differences on produced mussels products between tested culture depths were carried out using Two way-ANOVA for homogenous variances and Tuckey's test as post hoc test, or Kruskal-Wallis and Mann-Whitney U test for heterogeneous variances. Arcsine transformation (Zar, 1984) was applied to the data, expressed as percentage. No equal size samples were compared on both studied culture depth (number of "small" and "large" product) and thus, sum of square type IV was used (Zar, 1984). The signi□cance level was set at p=0.05 (Zar, 1984).

RESULTS

Production

Growth in weight of individual mussels and the loss of mussels observed in both culture depths was used to estimate biomass and production. Both values were estimated as Ash Free dry meat weight (AFDMW). Biomass is presented as *g* AFDMW per individual mussel as well as per meter. Production is presented as *g* AFDMW per meter and month.

Table 1 shows the seasonal mean biomass value ($g \cdot ind.^{-1}$). There was a peak increase in the mean biomass of mussels from winter (March 2014) to summer (August 2014). Mussels cultured at 5m depth showed to reached a peak in mean biomass from $0.172 g \cdot ind.^{-1}$ in winter to a mean biomass of $1.333 g \cdot ind.^{-1}$ in summer. Similarly, mussels cultured at 15m depth showed to reach a peak in mean biomass from $0.206 g \cdot ind.^{-1}$ in winter to a mean biomass of $1.007 g \cdot ind.^{-1}$ in summer. Higher total biomass increment over the experimental period was observed in mussels cultured at 5m depth ($1.319 g \cdot ind^{-1}$) compared to mussels cultured at 15m depth ($0.992 g \cdot ind.^{-1}$).

The seasonal biomass per meter of rope ($g \cdot m^{-1}$) showed the seasonal trend in AFDMW as well as the changes observed in population density in both culture depths. Seed from the same batch was used to test both

culture depths and the initial biomass was estimated as 10.035g·m^{-1} (Table 2). Two different trends in biomass per meter of rope (g·m^{-1}) were observed at each culture depth.

From seeding, in late spring, until fall, ropes cultured at 5m depth showed an increase in individual mean biomass of mussels (g·ind.$^{-1}$) cultured as well as an increase in the mean number of mussels per rope (n·m^{-1}). The number of new recruits observed at 5m depth resulted in a 1000 % increase in the mean biomass per meter of rope (g·m^{-1}) cultured at that depth. The increase in individual mean biomass (g·ind.$^{-1}$), however, was lower than those cultured at 15m depth. Ropes cultured at 15m depth showed higher mean individual biomass (g·ind.$^{-1}$) but a decrease of 38% of the number of individuals (n·m^{-1}). This resulting in a nearly 40 % lower mean biomass per meter of rope (g·m^{-1}) cultured at 15m depth compared to ropes cultured at 5m depth.

From fall (October 2013) to winter (March 2014) a decrease was observed in the mean number of individuals (n·m^{-1}) at 5m depth. Moreover, mussels at this culture depth showed a lower increase in mean individual biomass (g·ind.$^{-1}$) than those cultured at 15m depth. Ropes cultured at 15m depth showed a slight increase in the mean number of mussels (n·m^{-1}) as well as a higher individual mean biomass (g·ind.$^{-1}$). As a result of these opposite trends observed in winter (March 2014), ropes

from both culture depths displayed a similar mean biomass ($g \cdot m^{-1}$).

Form winter (March 2014) to summer (August 2014), an increase in the number of individuals ($n \cdot m^{-1}$) cultured in 5m depth was observed, whereas a decrease in the number of individuals ($n \cdot m^{-1}$) was observed at 15m culture depth. In addition, from winter to summer the trend of mean individual biomass ($g \cdot ind.^{-1}$) changed. Individual mean biomass ($g \cdot ind.^{-1}$) was higher at mussels cultured at 5m depth. At the end of the study in summer (August 2014), ropes cultured at 15m depth showed a final mean biomass per meter ($g \cdot m^{-1}$), 23% of the mean biomass per meter observed in ropes ($g \cdot m^{-1}$) cultured at 5m depth (Table 2).

Production, as defined by Stirling and Okumus (1995), is the total elaboration of biomass, and yield is the total biomass increment remaining on ropes. Temporal changes observed in production ($g \cdot m^{-1} \cdot mo^{-1}$) followed the changes observed in biomass per meter of rope ($g \cdot m^{-1}$). The highest production increment was also observed from winter (March 2014) to summer (August 2014) at both depths, with a value of 112.026 $g \cdot m^{-1} \cdot mo^{-1}$ at 5m depth and 39.238 $g \cdot m^{-1} \cdot mo^{-1}$ at 15m depth. The decrease in production observed from fall (October 2013) to winter (March 2014) at 15m depth was related to a lower growth rate and principally to losses and mortality.

The accumulation of net production is shown in Table 2. The

Table 1 Ash-free dry weight biomass and computation of production of experimental mussels in the submerged pilot structure for mussel growth between June 2013 and August 2014 at the two cultured depths (5m and 15m depths) test during 431 days study; where, Duration (number of days from seeding), N (mean number of individual mussels); AFDMW (mean ash free dry meat weight; g/per individual mussel); Production (g $\cdot m^{-1} \cdot mo^{-1}$) and Eliminated Biomass ($g \cdot m^{-1} \cdot mo^{-1}$).

Sampling time	Culture depth	Duration (days)	N ± SD (m)	AFDMW Individual Biomass (± SD; g)	Mean N (m)	Change in Biomass ($g \cdot ind.^{-1}$)	PRODUCTION ($g \cdot m^{-1} \cdot mo^{-1}$)	*Eliminated Biomass* ($g \cdot m^{-1}$)
13-Jun			681 ± 37	0.015 ± 0.012				
		119	*Average performance between sampling*		950	0.083	19.813	-7.611
13-Oct			1219 ± 424	0.097 ± 0.070				
	5 m	157	*Average performance between sampling*		820	0.075	11.732	20.605
14-Mar			420 ± 442	0.172± 0.110				
		155	*Average performance between sampling*		499	1.161	112.026	-22.914
14-Aug			577 ± 123	1.333 ± 1.127				
13-Jun			681 ± 37	0.015 ± 0.015				
		119	*Average performance between sampling*		471	0.165	19.622	10.285
13-Oct			262 ± 82	0.180 ± 0.073				
	15 m	157	*Average performance between sampling*		296	0.026	1.46	-2.552
14-Mar			331 ± 308	0.206 ± 0.208				
		155	*Average performance between sampling*		253	0.801	39.238	18.288
14-Aug			175 ± 76	1.007 ± 0.772				

turnover ratio of the net biological production to mean biomass (P:B) was 2.96 and 3.82 for ropes cultured at 5m and 15m depths, respectively.

In the present study new recruits not only obscured the quantification of the decreases in production from 5m depth culture scenario but also generated new biomass increments through a spontaneous settlement on the ropes. Therefore, it is suggested that the negative values found as EB (eliminated biomass) (Table 2) may be considered and equilibrated with a biomass of new recruits per meter to be added as a type of annually expected gained biomass per meter of rope, when considering the gross biological production in this area.

Nevertheless, as a result of these gained biomass, the real loss of "*in situ* production" (Table 2) of mussels remaining on ropes could not be estimated. These "*in situ* production" are reserve tissue losses used by the mussel to fullfil their metabolism and reproduction, not mortality measurements. Adding these seasonal decreases in production to the net biological production, the gross biological production is estimated.

The yield at 5m culture depth represented 105.67% of the net and the gross production. At least 5.7 % of the gross production was gained as a result of the recruitment observed in this culture scenario. The yield at 15m culture depth represented 57.69 % of the net production and the gross production. Hence, it can be deduced that 42.31 % of the gross

Table 2 Summary of biomass, eliminated biomass and production, as grams of ash-free dry weight per meter of cultivation rope (AFDW $g \cdot m^{-1}$) of mussels cultured at the two culture depths after 14 months of culture period.

	5m depth	15m depth
Initial biomass	10.04	10.04
A. Mean biomass	242.70	75.37
B. Final biomass	769.57	176.31
C. Biomass increment or yield (B- Initial)	759.54	166.28
D. Total eliminated biomass	-40.75	121.93
E. Cumulative net production (C+ D)	718.79	288.20
F. Production biomass ratio P:B (E/A)	2.96	3.82
G. % Retained as yield (100 *(C/E))	105.67	57.69
H. Total lost in situ production	0.00	0.00
I. Gross production (E + H)	718.79	288.20
J. % in situ loss (100 * (H/I))	0.00	0.00
K. % Yield (100 * (C/I))	105.67	57.69
L. % Total lost production (100-K)	-5.67	42.31

production at 15m depth culture scenario was lost due to drop-off and natural mortality. Nevertheless, this decrease could also be due to gamete production or due to the utilization of reserves during unfavourable periods. Hence, the loss of "*in situ* production" could be considered to be higher at 15m depth than at 5m depth. The yield at 15m depth was 40.10 % of that at 5m depth. However, net production was 54.56 % of that observed at 5m depth due to the higher recruitment observed at this upper depth.

Growth

The growth rates of *Mytilus galloprovincialis* Lmk. seeds (original seed batch), cultured at two culture depths in the SE Bay of Biscay, was assessed based on length-frequency data from sampling dates.

The measurements of shell length were grouped into 10 mm length-class ranges (constant size classes) and all the resulting length-frequency tables (Fig. 2) were resolved into Gaussian components (Fig. 3). The study identified a bimodal length distribution at 5m depth (classes I and II) and a unimodal length distribution at 15m depth (class I) (Figure 4). These pseudo-cohorts (classes I and II) were assumed to represent separate mussel batches. It was assumed that only the smallest mussel groups (i.e, individuals with a seed size shell length) would represent a separate "relative" age-class from the original batch.

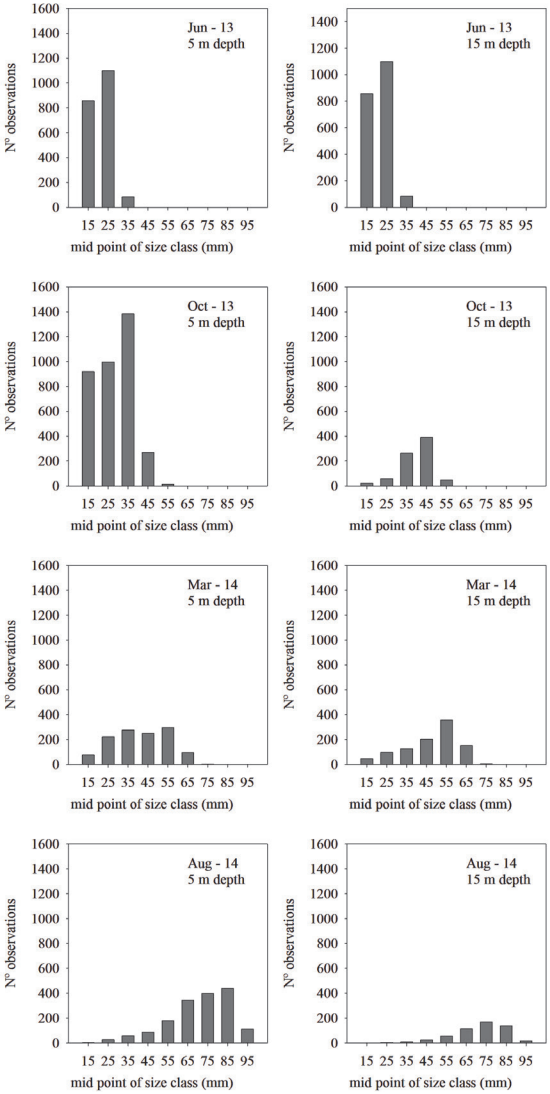

Figure 2 *Absolute frequencies (bar charts) found in Mytilus galloprovincilais (Lmk) at 5m and 15m depth at each sampling time: early-summer (June 2013), fall (October 2013), winter (March 2014) and summer (August 2014).*

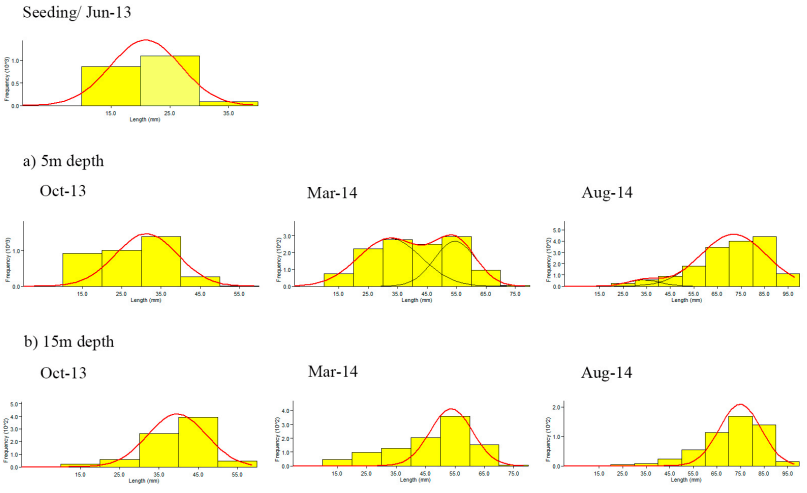

Figure 3 *FiSAT ouput of Bhattacharya method used to decompose composite length-frequency distributions (bar charts) found in Mytilus galloprovincilais (Lmk) at (A.) 5m depth and (B.) 15 m depth at each sampling time: a) June 2013, b) October 2013, c) March 2014 and d) August 2014. Iterative normal Gaussian function (red line).*

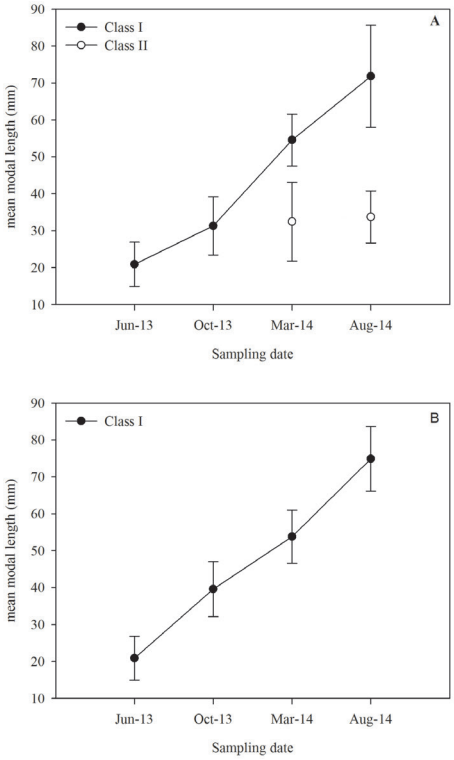

Figure 4 *Linking of modal lengths of Mytilus galloprovincialis (Lmk) found from seeding (June 2013) till harvest (August 2014) at 5m (A) and 15m (B) depths.*

A growth model was then fitted at each sampling depth to the presumed principal age-class (class I), original seed batch. Growth parameters of the Von Bertalanffy Growth Function (VBGF) were estimated with ELEFAN I from the growth increment data created by the linking of modal mean lengths (resulting in a "relative" size-at-age data).

Von Bertalanffy Seasonal Growth Function (VBSGF) parameters for *Mytilus galloprovincialis* (Lmk) growth at Mendexa, Bay of Biscay, based on length-frequency data from Bhattacharya method and ELEFAN I routine (FiSAT sofware). The maximum length observed at each culture depth was used as $L\infty$ (mm), which is the model asymptote for average length. The von Bertalanffy growth function estimated in this study to calculate growth rate for seeded mussels was that described by Somers (1988) (VBSGF) and applied accordingly:

5 m depth:

$L(t)= 96.73(1-e)^{-0.87(t- t0) - S(t) + S(t0)}$,

$S(t)= (0.8 \cdot 0.87/2\ \pi)\ \sin\ (2\ \pi(t - t_s))$,

$S(t_0)= (0.8 \cdot 0.87/2\ \pi)\ \sin\ (2\ \pi\ (t_0 - t_s)$

WP= ts +0.5=0.83

$t_s = 0.33$

t_0:

SS= 0.5 (June)

SL= 20mm

Rn = 0.940

15 m depth

$L(t)=93.52(1-e)^{-1.1(t- t0) - S(t) + S(t0)}$,

$S(t)= (1 \cdot 1.1/2\ \pi)\ \sin\ (2\ \pi(t - t_s))$,

$S(t_0)= (1 \cdot 1.1/2\ \pi)\ \sin\ (2\ \pi\ (t_0 - t_s)$

$WP= t_s +0.5=0.83$

$t_s=0.33$;

t_0:

SS= 0.5 (June)

SL= 20mm

Rn = 0.646

The final FiSAT length-frequency histograms and von Bertalanffy growth function output for mussel, *Mytilus galloprovincilais* (Lmk), cultivated at 5m and 15m depths are shown in Figure 5.

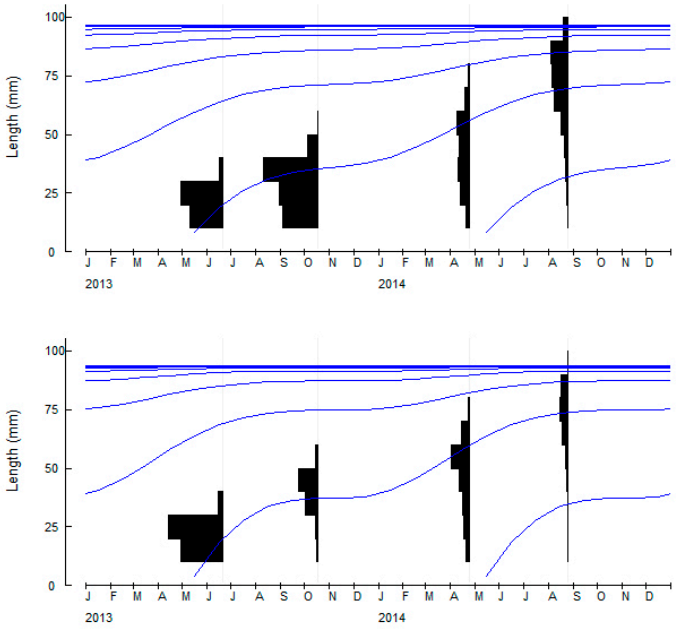

Figure 5 *Length-frequency histograms (bar charts) and Von Bertalanffy seasonal growth function (blue line) output from FiSAT for mussel, Mytilus galloprovincilais (Lmk), cultivated in Mendexa at 5m (A) and 15m (B) depths from June 2013 to August 2014.*

Commercial sizes at harvest

With respect to length-frequency distribution, the relative frequencies of individuals above 50mm shell length per rope and per tested culture depth is shown in Table 3. Almost half (46 %) of the mussels cultured at 5m depth reached the minimum commercial length (>50 mm shell length) after 9 months of culture. Similarly, after 9 months of culture nearly 60 % of the mussels cultured at 15m depth reached that size. At the end of the study, after 14 months of culture, nearly 90 % of mussels were above this minimum commercial size. Regarding differences in the production of the two commercial mussel products available ("small mussels" and "large mussels") at harvest, no significant differences were found between both culture depth scenarios (Mann-Whitney test, $p>0.05$).

Table 3 Percentage of "small mussel" product (50-70mm shell length), and " large mussel" product (≥ 70mm shell length) harvested at each sampling time and cultured depth (5m and 15m depths). No individuals below the minimum commercial size (<50mm shell length) were included in the present analysis.

	Oct-13		Mar-14		Aug-14	
(mean ± SD) %	5m depth	15m depth	5m depth	15m depth	5m depth	15m depth
Small mussel	0.43 ± 0.20	6.29 ± 3.02	46.04 ± 24.13	58.76 ± 14.15	32.42 ± 22.71	28.99 ± 10.06
Large mussel	0	0	0.38 ± 0.22	0.91 ± 0.61	56.62 ± 31.82	64.24 ± 9.36
Total	0.43 ± 0.20	6.29 ± 3.02	46.42 ± 24.29	59.67 ± 14.75	89.04 ± 3.02	93.24 ± 0.73

Product quality

Condition indexes

Condition index was calculated for mussels above minimum commercial length. Commercial mussel products were grouped in "small mussels" (50-70mm) and "large mussels" (≥ 70 mm) for testing. The observed mean ± SD of Freeman ś condition index values of these two commercial mussel products are shown in Figure 6.

Regarding seasonal effect on "small mussels" product condition index, Freeman CI was significantly affected by seasons at 5 m depth (Mann-Whitney test; $p<0.000$), whereas Freeman CI was not affected by seasons at 15 m depth (Mann-Whitney test; $p= 0.103$). Higher values were observed in summer sampling (August 2014). Regarding differences on "small mussels" product condition index due to culture depth, both winter mussels displayed similar CI value (Mann-Whitney test; $p =0.385$). On the contrary, in summer (August 2014) condition index were significantly affected by culture depth (Mann-Whitney test; $p=0.001$). Higher values were observed in mussels cultured at 5 m depth.

Regarding "large mussel" product, condition index of mussels were significantly affected by culture depth (Mann-Whitney test; $p<0.000$). Higher values were observed in mussels cultured at 5 m depth.

When condition of "small mussels" and "large mussels" products were compared (summer sampling), significant differences on condition index were observed with respect to size (Mann-Whitney test; $p<0.000$). "Large mussels" displayed higher condition than "small mussels". In addition, mussels cultured at 5m depth displayed significantly higher condition than mussels cultured at 15m depth.

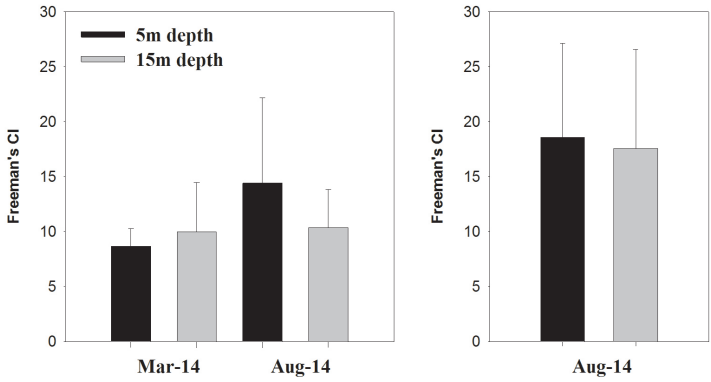

Figure 6 *Change in mean (± SD) of Freeman's condition index (CI) and Ash Free dry weight Condition index (AFDW CI) found in mussels cultured at 5 m depth (black bars) and cultured at 15m depth (grey bars) in "small mussel" product (A and B) and in large mussel product (C and D).*

Biochemical composition

Commercial mussel products were grouped in "small mussels" (50-70mm) and "large mussels" (≥ 70 mm) for testing.The observed values in biochemical composition of pools of mussel tissue from both mussel products per depth/sampling date are shown in Table 4.

Table 4 Proximate composition based on inorganic (Ash %), lipid (%), protein (%) and carbohydrates (%) on dry weight, and moisture (water %) from *Mytilus galloprovincialis* Lmk. mussel pools of different mussel product ("small" and "large" mussel products) harvested at each sampling time (March and August 2014at (a) 5 m and (b) 15 m culture depths.

Sampling Time	Mussel Product	Depth	Dry weight %				Moisture %
			Protein %	Lipid %	Ash %	Carbohydrates%	
14-Mar	small	5 m	52.21 ± 5.56	5.10 ± 1.30	19.36 ± 4.19	23.34 ± 7.13	86.04 ± 2.80
		15 m	53.95 ± 3.49	5.82 ± 2.72	18.11 ± 1.36	22.12 ± 4.63	85.33 ± 1.49
14-Aug	small	5 m	49.79 ± 7.44	5.59 ± 1.14	11.74 ± 1.75	32.88 ± 9.69	80.44 ± 2.02
		15 m	51.73 ± 5.94	5.67 ± 0.92	13.12 ± 1.50	29.48 ± 8.21	81.87 ± 1.14
	large	5 m	52.62 ± 4.47	7.53 ± 1.26	11.32 ± 1.22	28.53 ± 5.83	79.82 ± 1.71
		15 m	53.58 ± 5.92	7.54 ± 1.47	12.33 ± 1.50	26.55 ± 6.66	80.96 ± 1.46

In general terms, the following seasonal pattern was observed in "small mussels" sampled in winter and summer: (i) there was an slight increase in protein content (Two-way ANOVA; p=0.338) and in lipid content (Mann-Whitney U *test*; p= 0.485 at 5 m depth, and p= 0.818 at 15m depth) in summer (August 2014). (ii) Carbohydrate levels were significantly higher in summer (Two-way ANOVA; p=0.001). (iv) Ash content was significantly higher in winter (March 2014) at both culture depths (Mann-Whitney U *test*, p=0.004 at 5m depth and p=0.002 at 15 m depth). (v) Water content was significantly higher in mussels in winter (March 2014) (Two-way ANOVA; p<0.000).

No significant differences in gross biochemical composition were observed in "small mussel" product group in winter and summer samplings, with respect to culture depth (Two-way ANOVA; p>0.05). Similarly, no significant differences in gross biochemical composition were observed in "large mussel" product group sampled in summer, with respect to culture depth (One-way ANOVA; p>0.05).

Finally, when the biochemical composition of both mussel products ("small mussels" and "large mussels") harvested in summer (2014) were compared only lipid percentage was significantly higher in "large mussels" (Two-way ANOVA; p<0.000). Ash and water content percentages were higher in "small mussels" although significant differences were

not observed (Two-way ANOVA; p=0.224 and p=0.287, respectively). Regarding differences due to culture depth, ash and water content in mussels cultured at 15m depth was significantly higher than in mussels cultured at 5 m depth (Two-way ANOVA; p=0.042 and p=0.045, respectively).

Fatty acids

In total, 34 fatty acids were identified. The observed mean ± SD values of these FA contents of both commercial mussel products harvested per depth/season are shown in table 5. Most of the FA found in "small mussels" underwent seasonal changes during the studied period.

Regarding "small mussel" product (50-70mm), SAFAs, MUFAs, and PUFAs accounted for 25, 12, and 65 % of the total fatty acid composition found in these mussels, respectively. SAFA and MUFA fatty acids content varied significantly with seasons (Two-way ANOVA, $p<0.01$), whereas PUFA content did not (Two-way ANOVA, $p= 0.058$). SAFAs and MUFAs content increased from winter to summer, while PUFAs content decreased slightly in summer. SAFA content varied significantly with culture depth (Two-way ANOVA; $p<0.000$), whereas MUFA and PUFA content did not vary with culture depth (Two-way ANOVA; p=0.921 and p=0.893).

Regarding “large mussel” product (>70mm), SAFAs, MUFAs, and PUFAs content accounted for 28, 12, and 60% of the total fatty acid composition found in these mussels, respectively. “Large mussel” product was only harvested during summer (August 2014), and thus, no seasonal comparison was made. No significant differences in the content of these major fatty acids groups of “large mussels” were observed (Mann-Whitney test; $p>0.05$) with respect to culture depth.

When the levels of these major FAs groups were compare significant differences in SAFA and MUFA contents were observed between both mussel products cultured at 15m depth (Mann-Whitney test; $p=0.001$ and $p=0.043$, respectively), whereas no significant differences were observed between mussel products cultured at 5m depth (Mann-Whitney test; $p=0.181$ and $p=0.181$, respectively). No significant differences were observed in PUFA content between mussels products cultured in either culture depth (Mann-Whitney test; $p=0.529$ and $p=0.081$ at 5m and 15m depth, respectively).

For both mussel products (“small mussels” and “large mussels”), palmitic acid (16:0) was clearly the predominant SAFA, followed by stearic (18:0), and by myristic (14:0) acids. Gondoic acid (20:1 ω9) was the most abundant MUFA. The second major MUFA was palmitoleic acid (16:1 ω7) in winter (March 2014) and oleic acid (18:1 ω9) in summer (August

2014). Regarding PUFAs, both mussel products showed similar results with docohexaenoic acid (DHA) (22:6 ω3) as the most abundant fatty acid, and hexadecatetraenoic acid (16:4 ω3), followed by eicsapentaenoic acid EPA (20:5 ω3), as the second and/or third major PUFA.

There was a seasonal and an inverse fluctuation of EPA and DHA content. The DHA/EPA ratio in "small mussel" product was significantly lower in winter (March 2014) than in summer (August 2014) (Two-way ANOVA; p=0.011). No significant differences, however, were observed due to culture depth (Two-way ANOVA; p=0.102). Similarly, no significant differences in the DHA/EPA ratio were observed due to culture depth in "large mussel" product (One-way ANOVA; p=0.953). When "small mussels" are compared to "large mussels" significant differences in the DHA/EPA ratio were observed at both culture depths (Mann-Whiteny test; p=0.018 and p=0.003, respectively). "Small mussels" showed lower DHA/EPA ratio.

A high level of the ω3/ω6 ratio (above 7) was observed in all mussels above commercial size. The ratio in "small mussels" was significantly affected by season (Two-way ANOVA; p=0.002), but it was not affected by culture depth (Two-way ANOVA; p=0.191). The ratio in "large mussels" was significantly affected by culture depth (Mann-Whitney test; p=0.036). Nevertheless, no differences in ω3/ω6 between mussel

products were observed in either culture depth (Mann-Whitney test; p=0.066 and p=0.852, respectively).

Significant differences in the content of some principal FAs were observed. Among SAFAs, margaric acid (17:00) and stearic acid (18:0) were significantly higher in "small mussels" (Two-way ANOVA; p=0.016 and p=0.049), whereas Arachidic acid (20:0) and behenic acid were significantly higher in "large mussels" (Two-way ANOVA; p=0.022 and p=0.004). Regarding MUFAs, tetradecenoic acid (14:1), heptadecenoic acid (17:1), gadoleic acid (20:1ω11) and gondoic acid (20:1ω9) were significantly higher in "small mussels" (Two-way ANOVA; p<0.05), whereas palmitoleic acid (16:1ω7) and eicosenoic acid (20:1ω7) were significantly higher in "large mussels" (Two-way ANOVA; p=0.028 and p<0.000). Finally, regarding PUFAs, hexadecatetraenoic (14:4ω3) and arachidonic (20:4ω6) acids were significantly higher in "small mussels" (Two-way ANOVA; p<0.05), whereas α-linoleic (18:3ω3), stearidonic (18:4ω3), eicosadienoic (20:2ß) and eicosapentaenoic EPA (20:5ω3) acids were significantly higher in "large mussels" (Two way ANOVA; p<0.05). Adrenic acid (22:4ω6) was significantly higher in "small mussels" cultured at 15m depth (Mann-Whitney test; p=0.020) and docosahexaenoic DHA (22:6ω3) was significantly higher in "large mussels" cultured at 15m depth (Mann-Whitney test; p=0.008). The

content of essential fatty acids (EFA) (i.e., ∑(ARA + EPA + DHA)) was significantly higher in "large mussels" cultured at 5m depth (Mann-Whitney test; p=0.018) but no such difference was observed in mussels cultured ta 15m depth (Mann-Whitney test; p=0.059).

Table 5 Fatty acid composition (% of total fatty acids) of mussels (*Mytilus galloprovincialis* Lmk.) of "small mussel" product harvested in March 2014 and in August 2014, as well as "large mussel" product harvested in August 2014 at both culture depth levels (5m and 15m depths) tested. * Represent statistical significance in spatial (culture depth) pair-wise comparison for each mussel product and sampling time (t-test). Statistical significance was set up at ($p < 0.05$).

	SMALL				LARGE	
Fatty acids	Winter 2014		Summer 2014		Summer 2014	
	5m depth	15m depth	5m depth	15m depth	5m depth	15m depth
14:0	1.67 ± 0.27	1.63 ± 0.15	2.47 ± 0.35	2.34 ± 0.24	2.70 ± 0.54	2.46 ± 0.41
15:0	0.53 ± 0.06	0.59 ± 0.04	0.75 ± 0.03	0.78 ± 0.06	0.73 ± 0.07	0.75 ± 0.07
16:0	13.98 ± 1.50	14.14 ± 1.55	17.27 ± 0.83	16.95 ± 0.90	17.58 ± 2.08	17.95 ± 0.96
17:0	2.46 ± 0.45	2.42 ± 0.50	2.31 ± 0.20	2.55 ± 0.12	2.08 ± 0.35	2.27 ± 0.25
18:0	3.95 ± 0.18	4.04 ± 0.18	4.01 ± 0.26	3.92 ± 0.38	3.94 ± 0.28	4.28 ± 0.43
20:0	0.02 ± 0.04	0.03 ± 0.05	0.02 ± 0.03	0.01 ± 0.03	0.06 ± 0.02	0.03 ± 0.04
22:0	0.07 ± 0.17	n.d.	0.01 ± 0.01	n.d.	0.03 ± 0.02	0.01 ± 0.02
ΣSFA	22.68 ± 1.63	22.86 ± 1.90	26.85 ± 1.01	26.55 ± 1.23	27.12 ± 2.29	27.75 ± 0.87
14:1	1.39 ± 0.38	1.21 ± 0.23	0.67 ± 0.11	0.63 ± 0.08	0.56 ± 0.24	0.48 ± 0.06
16:1ω7	2.07 ± 0.42	1.82 ± 0.16	1.98 ± 0.30	1.87 ± 0.31	2.45 ± 0.47	2.15 ± 0.52
16:1ω5	0.18 ± 0.09	0.17 ± 0.09	0.28 ± 0.04	0.27 ± 0.02	0.30 ± 0.03	0.29 ± 0.03
17:1	0.84 ± 0.20	0.85 ± 0.16	0.50 ± 0.17	0.76 ± 0.08	0.41 ± 0.31	0.48 ± 0.21
18:1ω9	1.55 ± 0.29	1.51 ± 0.15	2.57 ± 0.24	2.56 ± 0.20	2.40 ± 0.53	2.30 ± 0.35
18:1ω7	1.22 ± 0.22	1.27 ± 0.16	1.24 ± 0.05	1.12 ± 0.05	1.27 ± 0.12	1.08 ± 0.42
20:1ω11	1.16 ± 0.23	1.22 ± 0.41	1.62 ± 0.11	1.80 ± 0.11	1.42 ± 0.14	1.50 ± 0.12
20:1ω9	3.24 ± 0.35	3.49 ± 0.26	3.37 ± 0.29	3.37 ± 0.33	3.01 ± 0.15	3.24 ± 0.15
20:1ω7	0.55 ± 0.04	0.55 ± 0.02	0.46 ± 0.04	0.48 ± 0.05	0.51 ± 0.03	0.56 ± 0.03
22:1	n.d.	n.d.	n.d.	n.d.	0.04 ± 0.04	0.02 ± 0.03
ΣMUFA	12.19 ± 1.26	12.09 ± 0.36	12.68 ± 0.51	12.87 ± 0.63	12.38 ± 0.74	12.09 ± 0.9
16:4ω3	12.24 ± 5.92	10.66 ± 5.15	10.04 ± 1.14	11.79 ± 1.64	8.82 ± 3.28	9.27 ± 2.31
18:2ω6	1.64 ± 0.14	1.53 ± 0.09	2.25 ± 0.12	2.36 ± 0.11	2.17 ± 0.29	2.18 ± 0.17
18:3ω3	1.90 ± 0.41	1.67 ± 0.20	1.71 ± 0.31	1.52 ± 0.13	2.04 ± 0.29	1.72 ± 0.2
18:4ω3	3.75 ± 0.90	3.25 ± 0.28	2.86 ± 0.90	2.44 ± 0.45	4.00 ± 0.82	3.20 ± 0.64
20:2α	4.54 ± 0.97	4.73 ± 1.88	3.80 ± 0.44	3.93 ± 0.31	3.27 ± 0.84	3.05 ± 0.24
20:2β	0.66 ± 0.13	0.69 ± 0.29	0.34 ± 0.05	0.33 ± 0.01	0.28 ± 0.21	0.26 ± 0.03
20:2ω6	0.76 ± 0.09	0.77 ± 0.05	0.66 ± 0.05	0.64 ± 0.03	0.73 ± 0.04	0.70 ± 0.08
20:4ω6	2.13 ± 0.27	2.43 ± 0.27	2.13 ± 0.35	2.28 ± 0.23	1.71 ± 0.26	1.83 ± 0.76
20:4ω3	0.52 ± 0.28	0.30 ± 0.05	0.21 ± 0.09	0.27 ± 0.15	0.36 ± 0.12	0.27 ± 0.07
20:5ω3	9.59 ± 0.85	9.63 ± 0.67	7.92 ± 0.59	7.41 ± 0.41	9.12 ± 0.40	9.06 ± 0.42
22:2 NMIDa	0.90 ± 0.10	0.95 ± 0.25	0.90 ± 0.10	0.95 ± 0.14	0.80 ± 0.09	0.71 ± 0.07
222 NMIDb	2.88 ± 0.43	3.22 ± 1.17	1.65 ± 0.21	1.73 ± 0.11	1.62 ± 0.78	1.38 ± 0.15
21:5ω3	2.18 ± 0.16	2.35 ± 0.57	1.44 ± 0.12	1.35 ± 0.07	1.42 ± 0.39	1.23 ± 0.06
224ω6	0.27 ± 0.03	0.34 ± 0.09	0.27 ± 0.06	0.45 ± 0.37	0.26 ± 0.04	0.25 ± 0.04
225ω6	1.14 ± 0.78	1.04 ± 0.33	0.86 ± 0.05	1.07 ± 0.33	0.87 ± 0.40	0.89 ± 0.09
22:5ω3	1.01 ± 0.05	1.08 ± 0.16	1.06 ± 0.04	1.06 ± 0.07	1.08 ± 0.06	1.10 ± 0.05
226ω3	19.03 ± 1.66	20.43 ± 1.38	22.36 ± 0.73	21.00 ± 0.88	21.93 ± 1.88	23.05 ± 1.25
ΣPUFA	65.13 ± 2.47	65.05 ± 1.95	60.47 ± 0.74	60.58 ± 1.69	60.50 ± 2.86	60.15 ± 1.36
NMID	3.78 ± 0.52	4.17 ± 1.42	2.55 ± 0.32	2.67 ± 0.23	2.42 ± 0.86	2.09 ± 0.21
ω3	50.21 ± 3.14	49.36 ± 2.59	47.60 ± 0.73	46.85 ± 1.40	48.79 ± 1.08	48.91 ± 1.51
ω6	5.93 ± 0.96	6.10 ± 0.51	6.17 ± 0.33	6.79 ± 0.36	5.74 ± 0.38	5.84 ± 0.78
ω3/ω6	8.67 ± 1.56	8.16 ± 0.95	7.73 ± 0.50	6.91 ± 0.32	8.53 ± 0.53	8.56 ± 1.62
DHA/ EPA	1.99 ± 0.14	2.12 ± 0.07	2.83 ± 0.23	2.84 ± 0.15	2.40 ± 0.13	2.55 ± 0.13
EFA	30.75 ± 2.49	32.48 ± 2.21	32.41 ± 0.92	30.69 ± 1.17	32.76 ± 2.05	33.94 ± 1.44
Diatom	0.15 ± 0.03	0.13 ± 0.01	0.11 ± 0.01	0.11 ± 0.02	0.14 ±0.02	0.12 ± 0.03
Brown algae	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.06 ± 0.05	0.03 ± 0.01	0.03 ± 0
Bacterial	0.79 ± 0.12	0.85 ± 0.07	0.48 ± 0.05	0.44 ± 0.03	0.55 ± 0.13	0.48 ± 0.19
Terrestrial	3.53 ± 0.54	3.20 ± 0.28	3.96 ± 0.40	3.87 ± 0.20	4.22 ± 0.58	3.90 ± 0.35
Detrital	4.21 ± 0.41	4.29 ± 0.60	4.30 ± 0.17	4.46 ± 0.15	4.09 ± 0.19	4.10 ± 0.5

FAs were also used as biomarkers of the mussel diet (Table 6). The bacterial trophic marker fatty acid ratio (18:1ω7 / 18:1ω9) in "small mussels" was not affected by season (Two-way ANOVA; p= 0.960), nor by culture depth (Two-way ANOVA; p=0.394). The bacterial trophic marker fatty acid ratio in "large mussels" was not affected by culture depth (One-way ANOVA; p=0.379).

The diatom trophic marker (16:1ω6/16:0) levels in "small mussels" was not affected by season (Two-way ANOVA; p=0.277) nor by culture depth (Two-way ANOVA; p= 0.602). The diatom trophic marker levels in "large mussels" was not affected by culture depth (Two-way ANOVA; p=0.091).

The brown algae trophic marker (20:4ω6/20:5ω3) levels in "small mussels" was not affected by season (Two-way ANOVA; p= 0.147) nor by culture depth (Two-way ANOVA; p= 0.170). The brown algae trophic marker levels in "large mussels" was not affected by culture depth (Two-way ANOVA; p=0.782).

The terrestrial trophic marker (18:2ω6 + 18:3ω3) level in "small mussels" was significantly higher in winter than in summer (Mann-Whitney; p= 0.002 and p=0.002 at 5m and at 15 m depth, respectively) but the level was not affected by culture depth (Mann-Whitney test; p= 0.937 and p=0.699 in winter and summer, respectively). The terrestrial

trophic marker level in "large mussel" product was not affected by culture depth (Mann-Whitney test; p=0.481).

The zooplankton trophic marker (18:1ω9) level in "small mussels" was significantly affectd by season (Two-way ANOVA; p<0.000) but not by culture depth (Two-way ANOVA; p=0.761). Higher levels were observed in summer (August 2014). The zooplankton trophic marker (18:1ω9) level in "large mussels" was not affected by culture depth (One-way ANOVA; p=0.646).

Regarding ω3 and ω6 FAs content, the content of ω3 FAs was slightly higher in "small mussels" cultured at 15m depth than in "small mussels" cultured at 5m depth (Two-way ANOVA; p=0.460). The content of ω6 FAs was slightly higher in "small mussels" cultured at 5m depth than those cultured at 15m depth (Two-way ANOVA; p=0.135). The content of both ω3 and ω6 FAs were slightly higher in "large mussels" cultured at 15m depth than in "large mussels" cultured at 5m depth (One-way ANOVA; p=0.851 and p=0.734, respectively).

When the level of these fatty acid trophic markers (FATM) between mussel products were compared, only terrestrial trophic marker levels were significantly different. "Small mussels" showed significantly higher content compared to that in "large mussels" at 15m depth (Mann-Whitney test; p= 0.003). Not such difference was observed between

mussel products cultured at 5m depth (p=0.066).

Table 6 Summary of the principal fatty acid trophic markers (FATM) used in this study to identify the food sources of mussels.

FATM	SOURCE	REFERENCE
16:1ω7 / 16:0 > 1 22:6ω3 / 20:5ω3 < 1	Diatoms	Budge et al. 2001; Dalsgaard et al. 2003
22:6ω3 / 20:5ω3 > 1	Dinoflagellates	Budge et al. 2001; Dalsgaard et al. 2003
20:4ω6 / 20:5ω3 >1	Brown algae	Kelly and Scheibling 2012
18:2ω6 + 18:3ω3 > 2.5	Terrestrial plants	Budge and Parrish 1998
18:1 ω9	Zooplankton	Kharlamenko et al. 2001
15:0 + 17:0 + 18:1 ω7	Bacterial / Detritus	Mayzaud et al. 1989; Najdek et al. 2002
18:1ω7 / 18:1ω9 > 1	Bacterial	Budge et al. 2001

DISCUSSION

Production and Biomass

There is little literature on the estimation of biomass and production of mussel *Mytilus* genera. Ceccherelli *et al.,* (1984) estimated it in wild populations, whereas Stirling and Okumus (1995) estimated it in cultivated mussels.

Production is determined by the balance of biomass increment over losses, which is affected by environmental and oceanographic variables found at each culture depth scenario as well as by the density of mussels on ropes.

This study found a continuous growth in length and in weight, and thus, production was always positive. This production, however, decreased at both depths under unfavorable conditions, found from fall to winter in our study area. In those unfavorable seasons mortality could not be correctly measured at 5m depth due to the presence of new recruits. At 15m depth, the production losses (fall-off as well as mortality) in both seasons were substantial.

Mortality is described as a negative change in abundance (or biomass) over some period of time. Mortality in mussel populations results from the interaction of various biological and physical factors (Dare, 1976). Seed source has a significant effect on populations mortality

(Fuentes *et al.,* 2000). Predation is also often suggested as the major natural mortality cause in bivalves (Walne and Davies, 1977; Diederich, 2006). Pérez-Camacho *et al.* (1991) found mortality rates between 2% to 36% from seeding to thinning out phases, and 15% from thinning out to harvest. Similarly, Fuentes *et al.* (1998) found rates between 4.5% to 43.8% from seeding to thinning out phases, and between 16,4% and 21.3% from thinning out to harvest. In this study, it was difficult to provide a correct estimation of mortality due to the high input of new recruits at 5m culture depth scenario, the lack of inspections due to bad weather conditions as well as the high temporal spaces between samplings. Nevertheless, at 15m culture depth the loss due to mortality, taking into account all culture phases from seeding to harvest, was approx. 42 %. Hence, the mortality rates found at the study site may well be similar to values found at sites were mussels are successfully produced.

The annual production of mussels at 5m depth was higher than at 15m depth because of a higher growth rate, higher final meat weigh and higher final density.

The ratio of net production to mean biomass (P:B), the turnover ratio, is used to estimate the production of mussel populations from their biomass. A net production to mean biomass ratio of 2.96 and 3.82 were observed in mussels cultured at 5m and 15m depths, respectively. These

values are slightly higher than the range of 1.8-2.7 previously recorded in some other cultivated mussels (Loo and Rosenberg, 1983; Stirling and Okumus, 1995).

The shell organic content was not measured in this study, and thus, the comparison with individual mean biomasses reported in literature, was not possible. This shell organic content could account for up to 3 % of the observed shell dry weight (literature value for mean shell organics; i.e., Dare and Davies, 1975). A calculated 3 % of the observed shell dry weight was added to estimate a new mean individual Ash Free shell dry weight (AFSDW). The mean AFSDW was added to the mean Ash Free Dry Meat Weight (AFDMW) in order to estimate a more accurate individual mean biomass. Hence, the new estimated (P:B) ratios were 2.89 and 3.65, for mussel cultured at 5m and 15m depth, respectively. The calculated new (P:B) ratios were still slightly higher than some previous observations (Rossemberg and Loo, 1983; Stirling and Okumus, 1995).

The maximum values of cumulative biomass were observed at the end of the study in summer (August 2014). The estimated turnover ratios (P/B) (2.89 and 3.65) corresponded to a turnover time (B/P*12) of approx. 4.2 and 3.3 months, respectively. Similar values of turnover time ranging from 3.5 to 11 months and concerning fast-growing populations were calculated in Dare and Davies (1975).

Growth

The length-frequency data from different seasons showed in winter (March 2014) and at the end of the studied period, summer (August 2014), a bimodal length distribution in the mussel population cultured at 5m depth. This bimodal distribution was not observed at 15m culture depth. The observed small shell length mode (i.e. presumed age-class II) may represent new recruits (different from the original batch). Spawning period is quite prolonged in this region (Ortiz-Zarragoitia *et al.,* 2011) and a recruitment in the open ocean pilot structure may have been occurred in late summer (seeding of the original batch was done in the end spring). This high increase of mussels of seed size was observed in fall (October 2013). Indeed, the number of mussels of seed size observed in fall were higher than the original number of mussels of that size seeded in spring (Table 1 and Figure 2). Thus, it is unlikely that all these small length mussels represent slow growing individuals from the original batch. In winter (March 2014) with the Bhattacharya method, the resolved modes enabled the separation of the two modes into separate age-classes (Separation Index (S.I.) = 2.5). Finally, in summer (August 2014), the S.I. between the two resolved modes was 3.65. The beginning of a new recruitment period could be expected in the pilot site from spring to late summer. Hence, these modes can also

be represented as separated "relative" age-classes. These mean modal lengths are based on the premise that because real age was not assessed, this principal cohort could therefore not disregard the presence of some fast-growing new settlers. Therefore, it would be prudent to consider this age groups as a pseudo cohorts.

The growth rates estimated in this study are based on the average growth of seeded individuals in the population (original batch) at the two culture depth scenarios tested. As individual growth variability is obscured, this hinders the correct deduction of the determinant environmental factors on growth (Bayne and Worral, 1980). Growth of ectodermic animals, such as invertebrate taxa, is seasonal, with high dependence on temperature and food supply (Pauly, 1990). To understand the effects of this seasonality on growth, it is essential to develop correct management practices for natural populations and/or extensive mussel production activities. During the present study, the mean growth rates of both populations were rapid from late spring to mid fall, slow during winter, and highest during summer. These observations are consistent with previous findings reported by Stirling and Okumus (1995) on mussel growth rates from Scottish waters.

The seasonal version of von Bertanlanffy growth function (VBGF), contained in ELEFAN I program, plotted the progression of modal

length-class ranges observed over time. This allowed the identification of the existence of a sinusoidal growth curve with marked oscillation over the year. In the studied area Summer point (T_s) was calculated as April 2013 at both culture depth scenarios. The amplitude of the parameter C indicates that over the course of the year, growth rate of mussels varies significantly. Hence, growth was significantly affected by seasons. This dependence was strong at both depths, albeit stronger at 15m depth.

This seasonality of growth could be related to the metabolic cost of different activities such as spawning (Pauly, 1979). The seasonal pattern of M. *galloprovicialis* Lmk at both culture depths showed highest growth rates in summer and lowest in winter, which seems not to be related to the pattern of primary production. Similar results in *M. galloprovincilais* (Lmk) growth were found by Fabi *et al.* (1989) in the Mediterranean Sea. This observed pattern can be partially explained by the influence of temperature on growth rate, as mussels grow more slowly in winter. Moreover, as suggested by Steffani and Branch (2003), mussel growth is faster at exposed sites as a result of greater water flow and currents which increases the availability of food per unit of time.

During winter, growth rate stabilizes and mussels grow more slowly. Mussels maintain their reached size during these slow growing months. In the studied site, harvesting may worth be done approximately in the

beginning of this slow growing period or, alternatively, during this slow growing period. Hence, as mussels size is stabilized during these months, harvesting along this period could be done regarding logistic reasons. In the studied case, mussels were seeded in early June and in October they hadn't yet reached minimum commercial size. In April, the Summer point of the studied population growth, this slow growing period would have finished and in fact, growth rate would be maximum until August. Due to the long time lag between samplings, harvesting management decisions cannot be exact. Similarly, this time lag enables the assessment of monthly increments in percentage of commercial sizes.

The environmental conditions observed in the studied area (Azpeitia et al., 2016) are corroborated with the growth observed in the VBSGF curves as well as with the observed condition index (CI) values. Hence, in order to harvest commercial size mussels with a CI above 10 in the studied site, it may well be predicted the benefits of seeding mussels in September and harvesting those mussels in the following September.

Mussel seeds needed 14 months of culture to reach a "large mussel" product size (≥ 70 mm). The results of this study are similar to those obtained for *M. galloprovincialis* (Lmk) at different culture sites (Table 7).

Mussel classification is based on the number of mussels of commercial length per kg (Peteiro *et al.,* 2006). The number of "large

mussel" product obtained at 5m depth was slightly lower than at 15m depth. Differences, however, were not notable. Moreover, the meat dry weight of commercial product was higher in mussels cultured at 5m depth and a higher asymptotic length value was also found at this depth. These would result in a better classification in terms of pieces kg^{-1} for 5m depth culture stock, with associated economic consequences.

Table 7 M. *galloprovincialis* growth at different geographic areas of cultivation literature data. Site Geographic area of cultivation, L0 initial mean size mm, Lt final mean size mm, t cultivation period in months.

Site	*Specie*	*L_0*	*L_t*	*t*	*References*
SW Bay of Biscay	*M. galloprovincialis*	18	50	5	Pérez-Camacho and Roman 1979
S Mediterranean Sea	*M. galloprovincialis*	10	40	12	Sarà *et al.* 1998
S Mediterranean Sea	*M. galloprovincialis*	43	63	12	Sarà *et al.* 1999
N Adriatic	*M. galloprovincialis*	46	69	12	Valli 1980
E Sicily	*M. galloprovincialis*	10	31	12	Genovese 1970
N Adriatic	*M. galloprovincialis*	Settlement	50	14	Ceccherelli and Rossi 1984
SE Bay of Biscay	M. *galloprovincialis*	20	72	14	This paper
N Adriatic	*M. galloprovincialis*	42	73	15	Fabi *et al.* 1989
SW Bay of Biscay	M. *galloprovincialis*	Settlement	82	16	Pérez-Camacho *et al.* 1991

Product quality

Mussel quality characteristics (i.e. condition, biochemical and fatty acids composition) as determined by seasonal intervals, can reflect the environmental conditions met by the animals at a specific time. Indeed, as reported by Orban *et al.,* (2002), the particular sessile habit of mussels makes their chemical composition strictly dependent on complex compensating factors (such as phytoplankton availability, climatic conditions, reproductive cycle, etc) which can vary seasonally as well as annually.

Condition index

Mussel condition in the studied area showed a seasonal cycle of tissue growth (particularly dry meat weight), which should be taken into account when marketing mussels (Stirling and Okumus, 1995). In the studied area the optimum time for harvest would be in summer (August 2014) as found by Orban *et al.* (2002). Harvesting one month either side of the expected spawning date is suggested (Stirling and Okumus, 1995). The spawning period on the Basque coast, as described by (Ortiz-Zarragoitia *et al.*, 2011; Azpeitia et al. submitted) is from spring to *late* summer. This spawning period is reflected with a decline in condition and hence meat quality in late fall and winter. Hence, mussels should be

harvested in late summer-early fall. Market demand for seafood in this region is linked to the winter season, which would force mussels to be harvested at this biological sub-optimal time.

"Small mussels" were harvested in winter and summer, and better condition was observed in the latter. Differences in condition observed with respect to culture depth did not stand out and therefore "small mussels" cultured at both depths could be mixed together for commercial purposes. In summer, both mussel products were harvested and "large mussels" displayed better conditions.

Biochemical composition and fatty acids

The proximate composition of mussels harvested at the experimental site underwent a seasonal biochemical cycle characterized by phases of accumulation and depletion of reserves in relation to their sexual cycle (Marthieu and Lubet, 1993). Gross biochemical composition of "small mussel" product followed a similar pattern to those observed by several authors (Beninger and Lucas, 1984; Orban et al, 2002, 2007).

The fluctuations of total lipids, glycogen, protein and ash content, reported on a dry basis in "small mussels", were found to depend on seasonality but not on culture depth. Moisture fluctuated, but differences were not observed with respect to depth. Protein content was higher

than glycogen content (34.80 ± 16.44 mg·g^{-1} at 5m depth and 33.88 ± 10.64mg·g^{-1} at 15m depth) during winter, as described by Zandee *et al.* (1980), and glycogen content (69.89 ± 26.55 mg·g^{-1} at 5m depth and 57.06 ± 19.81 mg·g^{-1} at 15m depth) was higher in summer. Several authors have observed high glycogen content during gamete proliferation or even preceding this gamete proliferation (Dridi *et al.,* 2007; Martínez-Pita *et al.,* 2012). Similarly, lipids are structural constituents of gonad membranes and are used as energy reserves for embryogenesis (Freites *et al.,* 2003; Gabbot, 1983; Ojea *et al.,* 2004 and Pazos *et al.,* 1997). In this study, lipids were higher in summer.

This lipid increase in summer is consistent with Prato *et al.* (2010) and very likely related to the storage cycle (Gabbot, 1983) of this species in the study area. From fall to spring lipids are used for gametogenesis, whereas protein and glycogen are used for both gametogenesis and energy production (Zandee *et al.,* 1980). The reproductive cycle of "small mussels" seem not to have altered as glycogen levels were high in summer.

Regarding "large mussel" product harvested in summer no differences were found in proximate composition with respect to culture depths. Similarly, no differences were found in the glycogen level (61.37 ± 17.59 mg·g^{-1} at 5m depth and 53.67 ± 17.13 mg·g^{-1} at 15m depth) with

respect to culture depths.

At the end of the study, in summer, the two mussel products from both culture depths presented similar proximate composition values than those described by Fuentes *et al.* (2009) in mussels (*Mytilus galloprovincialis* Lmk.) cultured at sheltered areas in the Galicia. Nevertheless, when both mussel products were compared, "large mussels" showed significantly higher lipid levels.

Seasonal variations in the diet composition of mussels were analyzed through the relative percentages of fatty acids and not in absolute terms. Hence, no quantitative assessment was attained.

Mussel food selection is not well understood. Mussels feed mainly on phytoplankton from seston, and this selection depends mostly on chemical cues, such as algal ectocrines (Ward and Target, 1989). Mussels may display useful mechanisms for phytoplankton sorting to ensure a complete exploitation of these resources, depending on seasonal variability in open waters. Phytoplankton communities vary seasonally in mussel habitats (Mackenzie, 1991), and mussels display seasonal changes in capture efficiency (Bayne *et al.,* 1977). Considering the physiological ecology of bivalve species, the selective ingestion of high-quality organic material is an important component of feeding physiology and scope for growth (Bayne *et al.,* 1989; Urrutia *et al.,* 1996; Hawkins *et al.,* 1998;

Bayne, 2002).

Selective ingestion of different algal species has been observed in different mussel species. Sidari *et al.* (1998) observed that *Mytilus galloprovincialis* Lmk. selects dinoflagellates rather than diatoms. Ren *et al.* (2006) demonstrated that Absorption efficiency (AE) of dinoflagellates as well as flagellates was generally higher than those of diatoms. Diatom cell walls are rigid and resistant to both enzymatic and physical breakdown. Moreover, diatoms contain significantly higher quantities of inorganic matter. A selective rejection of diatoms in the pseudofeces has also been documented (Shumway *et al.,* 1985), pointing out mussels' mechanism to sort phytoplankton. Newell *et al.* (1989), however, observed a prevalence of diatoms in the gut of *Mytilus edulis* L. In addition, Rouillon and Navarro (2003) demonstrated a selective diatom digestion when mussels were fed with diatoms and naked flagellates. These results are also consistent with studies which shows high correlation of mussel growth to diatom abundance compared to that with flagellate abundance (Beukeman and Cadee, 1991).

"Small mussel" product was harvested in winter and summer. These "small mussels" showed SAFAs, MUFAs and PUFAs proportions consistent with King *et al.* (1990), Frolov *et al.* (1995), Freites *et al.* (2002b), Alkanani *et al.* (2007) and Dridi *et al.* (2007). No spatial differences

were found, although seasonal changes in SAFA content (increasing in summer) and PUFAs content (decreasing in summer) were found. This is consistent with Fernández-Reiriz *et al.* (2015). These high levels of unsaturation, observed in winter, maintain membrane fluidity at lower temperatures (Thompson *et al.,* 1992; Hall *et al.,* 2002; Ezgeta-Balić *et al.,* 2012). Similarly, a decrease in the levels of 14:0, 16:0 and EPA (20:5ω3) FAs from winter to summer support the energetic role of these FA, which are catabolized to generate the energy for diverse metabolic functions (Freites et al 2002a).

In this study, the presence of NMID (22:2) was consistent with previous observations on *Mytilus galloprovncialis* Lmk. (Freites *et al.,* 2002a, Fernández-Reiriz *et al.,* 2015). Freites *et al.* (2002 b) described species-to-species variability in NMID FAs amount in mollusc, ranging from 0.7 to 20.7 %. NMID levels, in "small mussels", were higher in winter, when PUFA levels were high. Some authors described that PUFA deficiency induces the biosynthesis of NMID FAs (Fernández-Reiriz *et al.,* 1998; Ventrella *et al.,* 2008). Other authors, however, have described independent biosynthesis of NMID during high PUFA levels (Fernández-Reiriz *et al.,* 2015; Pirini *et al.,* 2007).

The predominance of phytoplankton class and other food sources, assimilated by mussels, is reflected by different trophic markers (Table

6). Change in DHA and EPA levels, major PUFAs, are related with seasonal phytoplankton class variability. Diatom and dinoflagellates contain considerable amounts of EPA and DHA, respectively (Chuecas and Riley, 1969). Higher levels of EPA, found in mussels in winter, may be related to the ingestion of dinoflagellates (Freites *et al.,* 2002 a), whereas the higher levels of DHA, found in mussels in summer, may be related to the ingestion of diatoms (Handå *et al.,* 2012).

Regarding "small mussels'" diet in winter and summer, diatom biomarkers (16:1ω7/16:0 ratio and ratio EPA/DHA) were below 1 in the present study in both winter and summer samplings. Higher values were found in winter. In addition, dinoflagellate biomarker (DHA/EPA) was above 1, which reflects a dinoflagellate-based diet. Higher values of this dinoflagellate biomarker were found in summer.

Budge *et al.* (2001) indicated that a value above 1 in the ratio of 18:1ω7/18:1ω9 FAs could indicate bacterial input in mussels' diet. In the present study bacterial biomarker ratio was below 1. This low bacterial input, however, was higher in winter and at 15m depth. A detrital/bacterial trophic marker (i.e., Σ(15:0 + 17:0 + 18:1ω7; Mayzaud *et al.,* 1989; Najdek *et al.,* 2002) with relative percentages ≥4 % is indicative of a bacterial input in the diet. Detrital/bacterial biomarker levels were constant from winter to summer.

FA trophic markers of terrestrial organic sources (i.e., Σ(18:2ω6 + 18:3ω3)) higher than 2.5 were described by Budge and Parrish (1998) as indicative of a terrestrial organic diet source. Highest levels were found in summer, although levels above 3 were also found in winter.

In conclusion, in the studied area "small mussels" in winter seemed to have fed mainly on dinoflagellates as well as on small proportions of bacterial and terrestrial organic sources. In summer, "small mussels" seemed to have fed on dinoflagellates but also on diatoms. They also have fed on a lower proportion of bacterial organic sources and a slightly higher proportion of terrestrial organic sources, compared to winter.

"Large mussel" product were only harvested in summer. SAFAs, MUFA and PUFA levels were similar in mussels of both culture depth scenarios. Palmitic (16:0) and gondoic (20:1ω9) acids were major SAFAs and MUFAs in mussels. These findings are consistent with Alkanani *et al.* (2007), Freites *et al.* (2002b) and Orban *et al.* (2002). Among PUFAs, DHA (22:6 ω3), hexadecatetraenoic acid (16:4 ω3) and EPA (20:5 ω3) were the most prevalent fatty acids. This is consistent with Abad *et al.* (1995) and with different studies from the Mediterranean (Karakoltsidis *et al.,* 1995), Atlantic (Fernández-Reiriz *et al.,* 1996), and Adriatic Sea (Orban *et al.,* 2002) which reported similar fatty acid profiles in the mussel *Mytilus galloprovincialis* Lmk. of this commercial product size.

The fatty acids profile of both mussel products ("small mussels" and "large mussels") harvested in summer differed significantly. "Large mussels" showed higher levels of essential fatty acids (EFAs) (i.e., Σ(ARA+ EPA+DHA)). "Large mussels" contained significantly higher relative percentage of EPA, whereas "small mussels" contained higher relative percentages of DHA and ARA. Similar results in EFAs levels between "small" and "large" *Perna canaliculus* mussel's FA signatures were observed by Taylor and Savage (2006).

Differences in the diet in summer of mussels from both mussel products at the study area were compared. The DHA/EPA ratio of "small mussels" was significantly greater than in "large mussels", suggesting "small mussels" may have assimilated a greater percentage of dinoflagellate microalgae (Taylor and Savage, 2006).

The ω3 FAs are selectively concentrated by bivalves, whereas when ω3 and ω6 PUFAs are available in similar proportions, ω6 PUFAs are not selectively concentrated (Ackman, 1983). No significant differences in ω3 FAs and ω6 FAs levels were observed between both mussel products. "Small mussels" cultured at 5m depth showed higher content of ω6 FAs and lower content of ω3 FAs than "large mussels". The opposite was observed in mussels cultured at 15m depth. The ω6 FAs level, was related to the levels of 18:2ω6, 20:2ω6, and 20:4ω6 FAs. The content of

these three FAs is presumably associated with a high intake of the first (18:2ω6 FA). This FA is likely to have been obtained from a microalgae diet (Freites *et al.,* 2002) by "small mussels" in this study area. These "small mussels" also showed significantly higher levels of 20:4ω6 FA. The levels of 20:2ω6 FA, however, were significantly higher in "large mussels".

Budge *et al.* (2001) suggested that the optimal ratio of ω3/ω6 PUFAs was between 5 and 15, for mussel growth. Herein, both mussel products showed a stable ω3/ω6 ratio, from 6 to 7 in "small mussels" and from 8 to 9 in "large mussels". The values are considered medium-high level concentrations. Mussels from exposed locations have more limited capability for *de novo* synthesis of PUFAs and acquire them from diet.

NMID FAs may have a structural-type function (Takagi *et al.,* 1980) and confer resistance to lipase microbial attack in the external membranes of the marine invertebrates (Ackman and Hooper, 1973). NMID levels were higher in "small mussels" although differences were not significant. The relative percentage of the precursor of NMID FAs, 16:1w7 (Zhukova, 1991), however, was significantly higher in "large mussels".

FAs such as 14:0, 16:1ω7, 18:1ω9, 18:4ω3, 20:5ω3, and the ratio ω3/ω6 PUFAs have mainly an energetic character (Freites *et al.,* 2002b).

"Small mussels" showed lower content of some of these energetic character FAs (14:0, 16:1ω7, 20:5ω3, as well as a lower ω3/ω6 ratio). Regarding diet, some diatom species are especially rich in 18:1ω9, 18:4ω3 and 20:5ω3 FAs (Ackman *et al.,* 1968; Fernández-Reiriz *et al.,* 1989). "Small mussels" showed significantly higher 18:1ω9 levels, whereas "large mussels" showed significantly higher levels of 18:4ω3 and 20:5ω3 FAs.

20:1ω9 FA is indicative of a carnivorous diet (Virtue *et al.,* 2000), whereas 16:1ω7 and 18:1ω7 FAs are indicative of phytoplankton dietary input. Carnivorous trophic marker was significantly higher in "small mussels", whereas higher levels of phytoplankton trophic markers were observed in "large mussels". Prato *et al.* (2010) found 4-6% of the carnivorous trophic marker (20:1ω9 FA), which is slightly higher than those observed in this study (2-4 %). Regarding phytoplankton trophic markers, differences were only significant for 16:1ω7 FA level. This FA (16:1ω7), through chain elongation, would derive in 18:1ω7 (Kharlamenko *et al.,* 2001). This indicates that mussels in the studied area have fed on zooplankton and that "small mussels" may have assimilated significantly more zooplankton than "large mussels".

In conclusion, in summer "small mussels" seemed to have assimilated dinoflagellates better than "large mussels". "Large mussels" also have

fed on dinoflagellates but they may have had a greater assimilation of diatoms than "small mussels". These "large mussels" also seemed to have assimilated terrestrial organic sources better. By contrast, "small mussels" seemed have assimilated zooplankton in greater proportions than "large mussels".

Conclusions

Mussel *Mytilus galloprovincilais* Lmk. was a natural choice, as this bivalve specie is native to this region, fast-growing and commercially viable (Azpeitia *et al.,* 2016; Azpeitia et al., 2017). Differences were found in the timing and duration of annual periods of growth, at two culture depth populations. Growth estimates identified overall different seasonal growth rates at both depths. As mussels used in this comparative growth study were from the same seed source, variation in growth could be attributed to environmental factors, as reported previously by Mallet *et al.* (1987), Fuentes *et al.* (2000) and Garen *et al.* (2004). Physiological studies suggested related a number of factors the observed growth differences; perhaps the most striking of these was temperature rather than the observed food availability (Azpeitia et al., 2016).

The present results are relative as the pilot estimation of mussel productivity on our coast is presented to determine the viability of commercial production. These kinds of studies should be linked to

mortality and recruitment studies for a comprehensive understanding of production, and finding differences in production with respect to culture depth. The implications of the present findings could be used for future aquaculture management in the region and for scientific experiments. The time lag between samplings was quite prolonged in this study and therefore harvesting management decision cannot be exact.

In terms of possible future production, if seeding is made in late spring (as the studied case) thinning out processes during late summer-fall could help to control density, to minimize eliminated biomass as well as to clean the ropes of new recruits that would compete for space and resources with the cultured batch. This thinning out could also be done in winter as nearly 45% of the population (at both depths) were within thinning out size ("small mussel" length; 50-70mm) in the present study. In addition, growth rate is stabilized in winter and thus product size won't change during this season. Moreover, the mussels harvested below minimum commercial length (≤50mm) could be reseeded separately for growth. The separation of mussels by their shell length would promote growth of culture mussels and improve the production yields as well as decrease the *in situ* mussel losses in a possible future scenario.

Thinning out processes, however, are time-consuming and labor-intensive. Thus, different seeding densities could be tested in order to

compare different scenarios with and without thinning out processes.

In the studied area the optimum time for harvesting mussels, seeded in the previous late spring, was in the following summer. In this case, however, harvesting should be delayed to late summer-early fall to avoid spawning periods.

In order to mitigate the problem of harvesting at sub-optimal time for market demand (winter), year-long processing facilities could be created as suggested by Peharda *et al.* (2007) which could enable sales throughout the year. Indeed, the clear seasonal growth curve observed at the studied site proves that year-round harvesting is possible.

At the studied site the optimum seeding time may well be in early fall (September), in which case, harvesting may well be optimum from the following early fall to the end of winter (i.e., in the slow growing period). Thus, in the studied site the culture period to produce mussels of commercial size will last a year after seeding, if the seeding is done in the aforementioned optimum time or alternatively, it will last one-and-a-half years, if seeding is done in sub-optimal time (as in the studied case). Nevertheless, once mussels attain their commercial size and reach the slow growing period, they could be harvested anytime in this period. In addition, during this slow growing period harvesting management decisions could be made based on logistic reasons. Despite this, mussel

condition changes seasonally and during part of the slow growing period mussels may display slightly different conditions and thus, the quality of the final product could change. These mussel products with different quality characteristics may well be bound to different type of market (e.g., canned, fresh). Similarly, different seeding times could also be studied in order to assess these expected temporal differences in production and biomass in year-round, continuous mussel production.

Differences in mussel classification (pieces of mussel product·kg^{-1}) between both culture depths were not notable and thus both culture depths could be harvested together for commercial purposes.

Beukema and Cadee (1991) point out that the precise knowledge of characteristics of natural diets available to bivalve populations, particularly the phytoplankton composition, is of prime importance for understanding population dynamics and mussel growth. This study is the first attempt to identify major food sources of *M. galloprovincialis* Lmk. cultured in the SE Bay of Biscay, using FA trophic markers to examine the extent of capability of *M. galloprovincilias* Lmk. to use available food sources. Mussel of different lengths develop different feeding strategies, which can be key factors in ecosystem functioning. For both studied mussel products, diet seemed to consist of various species of dinoflagellates, supplemented by a variety of diatoms,

bacteria, organic particles, flagellates and other protozoas, unicelular algae, as well as detritus consisting of particles from cytolised cells of a great variety of plants and animals.

Regardless of the feeding differences observed between mussel products, both products displayed similar quality characteristics. Regarding FA composition as a quality characteristic, significant differences were neither observed in DHA and EPA levels nor in the total ω3 FAs content, with respect to culture depth or product type. Hence, the manner in which mussels are harvested, and at which depth, will be based on logistical considerations. Furthermore, if cultured mussels are to be used for ω3 FA extracts, the two mussel products could be mixed.

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CHAPTER 6

A SENSORY AND NUTRITIONAL VALIDATION OF OPEN OCEAN MUSSELS (*Mytilus galloprovincialis* Lmk.) CULTURED IN SE BAY OF BISCAY (BASQUE COUNTRY) COMPARED TO THEIR COMMERCIAL COUNTERPARTS FROM GALICIAN RÍAS (SPAIN).

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SUMMARY

Mussel shell biometry, nutritional quality as well as consumer sensory evaluation of experimental open ocean cultured mussel *Mytilus galloprovincialis* (Lamarck 1819) were analyzed and compared to that of commercial mussels from Galician Rías available in the local market. Both mussel products were of the same commercial size. In this study, open ocean mussels were significantly higher and wider than those of Galician Rías. In addition, with the exception of ash content, both mussel products showed similar biochemical composition. Regarding fatty acid profiles, however, statistical differences were detected. These differences were not fully reflected in the sensory assessment. In terms of consumer acceptability, both mussel products were considered equally satisfactory.

Key words fatty acids, mussel, *Mytilus galloprovincialis*, open ocean aquaculture, proximate composition, sensory analysis.

INTRODUCTION

Aquaculture is a seafood supply that has increased gradually over the last few decades (Langan and Horton 2003). This growth is due to progress in technology, changes in policy, and an increased societal awareness of sustainability (Bushek 2004; Coen *et al.* 2007; Costa-Pierce 2008; Forrest *et al.* 2009; Bostock *et al.* 2010). In particular, mollusk aquaculture represents more than 75% (13.9 million tons) of the world's aquaculture, with mussel production representing around 13% (1.8 million tons) of its annual production (FAO 2014). Spain, France, and the Netherlands produce one third of the global mussel aquaculture production (Buck *et al.* 2010). Specifically, Spain, with an average production near 200,000 tons per year, is the second largest producer worldwide and the first in Europe (FAO 2014). A large part (95%) of its annual production is produced in the Galician Rías from the SW Bay of Biscay (Galician region). Since 2008, mussel products from that area have had a Protected Designation of Origin certifying quality and traceability within the standards of the EU seafood policy.

Shellfish species are a valuable food source in the socioeconomic context of the Basque Country (SE Bay of Biscay) (Gracia 1996), yet mussel aquaculture is limited. Indeed, over the last few decades, every mollusk (especially bivalves) consumed in this area has been imported.

The lack of development of an aquaculture or shellfish sector in the region of the Bay of Biscay is mainly linked to high wave-energetic scenarios (Galparsoro *et al.* 2012) and previous issues related to estuarine water-quality (Chust *et al.* 2009). A further restriction is current legislation, which aims to prevent conflict between users of marine habitats. These conflict-prevention methods hinder the development of near-shore aquaculture as the limited inshore spaces, are protected marine habitats (Galparsoro *et al.* 2010; Borja *et al.* 2011; Pascual *et al.* 2011).

In addition to these local limitations, the main obstacles in mussel aquaculture in general are those regarding infectious diseases and parasites (Bower *et al.* 1994), marine phycotoxins (Svensson 2003; Moroño *et al.* 2003) as well as carrying capacity and competition for the use of marine space (Martínez *et al.* 2007; Costa-Pierce 2008; Byron *et al.* 2011). Limited marine space coupled with commercial objectives often results in non-sustainable practices for commercial mussel cultivation (Smaal 2002; Waite *et al.* 2005; Duarte *et al.* 2008). It is encouraging, however, that ecosystem-based approach-management strategies have been recommended to promote the sustainable development of mussel cultivation (Soto *et al.* 2008).

One such strategy is open-ocean aquaculture which represents a promising alternative for the sustainable cultivation of bivalve species

(Polk 1996; Hesley 1997; Stickney 1998, Bridger and Costa-Pierce 2003). However, this activity is still in development stages (Hickman 1992), and main mussel culture techniques (i.e. rafts, pole racks and/or longline systems) have been established almost exclusively in protected near-shore waters (Pérez-Camacho *et al.* 1991; Myrand *et al.* 2009) or in estuarine habitats (Penney *et al.* 2002; LeBlanc *et al.* 2005; Drapeau *et al.* 2006). Therefore, few studies have reported successful biological and/or engineering results on open ocean mussel aquaculture to date (Langan 2001; Langan and Horton 2003; Buck 2007).

Open ocean aquaculture is not only a financially attractive but also an environmentally sound one with a highly nutritious end product for the consumer. Indeed, there is an increasing demand for products sourced from open ocean aquaculture methods, resulting in increased revenue in international markets (Orban *et al.* 2002; Fuentes *et al.* 2009; Pogoda *et al.* 2013). Further, developing open ocean aquaculture methods will be environmentally beneficial (Shumway *et al.* 2003), as mussels and other bivalves are filter feeders, capturing and eating microscopic particles, plants, and nutrients by filtering, and thus cleaning ocean water (Shpigel *et al.* 1993). For the consumer, as most farmed mussels have fast growth, they provide year-round nutritious food, much in demand (Seed and Suchanek 1992; Gosling 2003). Mussels are appreciated by humans for

their organoleptic properties (Grienke *et al.* 2014) as well as for their health benefits. They provide high-quality protein, minerals, essential trace elements, fat-soluble vitamins (i.e. vitamin D), and essential fatty acids EFA, especially long chained Polyunsaturated fatty acids; omega-3 (ω3 LC-PUFAs) (Stankovic *et al.* 2011; James 2013).

Regarding consumption, mussel shape and appearance are decisive factors for consumers' purchase (Fuentes *et al.* 2009), whereas meat quality (and hence, apparent health benefits) is regulated by biochemical composition (Orban *et al.* 2002; Filgueira *et al.* 2006; Fuentes *et al.* 2009). Mussel meat quality has been described by consumers as the result of not only chemical and biological characteristics, but also organoleptic properties, such as the appearance of the muscle, the intrinsic flavor, and the absence of undesirable components (Vernocchi *et al.* 2007). As reported by Fuentes *et al.* (2009) mussel organoleptic characteristics can be origin-dependent. This means that different production sites, with different conditions and culture technologies, are expected to promote changes in growth performance and biochemical composition of mussel individuals, which could in turn reflect different product qualities at consumer level (Oliveira *et al.* 2015).

To date, no studies have investigated organoleptic properties on open ocean mussel products from the SE Bay of Biscay. Both biochemical

composition and organoleptic properties are of special importance to anticipate consumer acceptance and market feasibility (Gökoglu 2002; Oliveira *et al.* 2015) before considering any implementation of mussel activity in the region.

The specific goals of this study were to characterize and compare the biometric parameters, nutritional content, and sensory aspects between experimental mussel product from the SE Bay of Biscay (open ocean) and commercial mussel product from the SW Bay of Biscay (Galician Rías).

MATERIAL AND METHODS

Location and sampling

An experimental suspension longline culture system (Langan 2000) was developed to cultivate shellfish in the open waters of the south eastern corner of the Bay of Biscay (Basque region; Spain). This region extends over 150 km and it is oriented E-W. The climate is temperate, oceanic, with moderate winters and warm summers. According to Köppen's classification, the area is associated with a marine temperate climate (Cfb) (Collins and Borja 2004). Seeds (17.40 ± 5.07 mm) were naturally collected during June 2013 and cultivated at the experimental site (43° 21.39'N; 02° 26.90'W) over a one year period. The open ocean site is already classified as a Class A production area (according to EU regulation (EC) 854/2004). Therefore, those mussels are not subjected to depuration requirements. The site is located at 2 nautical miles off the coast without any protection from land masses (Ryan 2004). Galparsoro *et al.* (2012) described strong hydrodynamic conditions on Basque Country's coast. A detailed description of current and waves in the experimental site can be found in Azpeitia *et al.* (2016) (chapter IV).

Mussel individuals were cultured within 17m water column depth, on mussel-growing ropes of 12m in length. Mussel ropes hung from 5m headline depth, with a distance between ropes of 0.5 meters and

an initial stocking density of 681 ± 37 individuals per lineal meter. After 13 months of cultivation, in July 2014, 200 mussel specimens of "commercial size" (Peteiro *et al.* 2006; i.e. 70 mm) were carefully detached from the ropes by cutting the byssal threads and cleaned of encrusting organisms. Selected mussel individuals were transported in plastic self-draining boxes covered by flaked ice. Later in the laboratory, mussels were conserved in plastic boxes, stored at 4ºC and covered with flaked ice for 24 hours previous to analysis in order to preserve them. Similarly, 200 mussel individuals from Ría de Arosa (Galician Region; Spain) with a comparative commercial size (69.65 ± 6.11 mm) were obtained at early hours on the morning of the day of the sensory analysis. Commercial mussels were bought from a local seafood retailer to ensure a comparable size product with the same conservation procedure (plastic box and stored at 4 ºC). These mussels were also stored at 4ºC and covered with flaked ice for some hours previous to analysis. Subsequently, two stocks of 50 mussel individuals from Galicia and from the experimental open ocean site were frozen at -80ºC to await morphometric and biochemical analysis. (Figure 1)

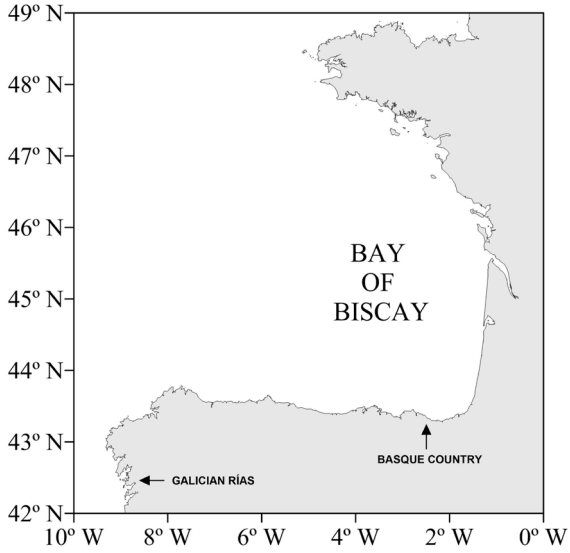

Fig 1 Map of the Bay of Biscay showing the specific location of the open ocean experimental site at the SE Bay of Biscay (Basque Country) and the commercial mussel product from the SW Bay of Biscay (Galician Region).

Morphometry

Twenty mussel individuals from each origin (experimental open ocean and commercial) were carefully detached by cutting the byssal threads, cleaned of encrusting organisms and measured for morphometric analysis. The individuals were measured for their shell length (L; maximum anterior–posterior axis), shell height (H; maximum

dorso-ventral axis), and shell width (W; maximum lateral axis) using a digital caliper to the nearest 0.1 mm. The biometric terminology used in this study is that described by Seed (1968). Mussel shape was analyzed by linear allometric relation between log-transformed mussel shell dimensions (length (L), height (H) and width (W)).

Biochemical composition & Fatty acids

Three replicate samples of 10 pooled mussel individuals from each origin were considered for biochemical characterization (including proximate and glycogen analysis) and lipids determination (including total lipids and fatty acids), respectively.

The fresh individuals were firstly minced in a food processor (IKA® M 20 universal mill, IKA 1603601, Germany). Moisture and ash were determined according to the gravimetric determination. Briefly, minced tissue pools were placed in pre-weighed porcelain trays for drying at 80°C for 24h and then weighed to the nearest 0.001 g. Subsequently, dry mussels' tissue pools were ashed at 450°C for 4h in a muffle furnace, cooled in a desiccator and weighed again to determine the ash-free dry weight content.

Total protein was determined according to the Kjeldahl's method (AOAC 1975). Samples were digested in a digestor BUCHI B-435, distilled using a Rotavapor® R-210 (BÜCHI Labortechnik AG, Switzerland) and

automatic volumetric titration developed through a Mettler Toledo T-50 (Mettler Toledo, United States).

Glycogen concentration ($mg \cdot g^{-1}$ mussels' tissue) was calculated based on a mussel glycogen standard (Sigma, Saint Louis, MO) following the methodology reported by Gallardi *et al.* (2014). Briefly, 30% KOH was added to 0.5 g of homogenized mussels' tissue (ratio 2:1). The samples were then heated in a shaking-water bath at 100°C for 20min, vortexed for 30s and subsequently chilled on ice for 5min. After cooling, 200 µL of each sample was transferred to a 4mL glass vial followed by 200 µL of 95% ethanol. The solution was vortexed again briefly and then placed in a boiling-water bath for 15min followed by the addition of 1.2mL of lukewarm water. The samples were again briefly vortexed and then allowed to stand at room temperature for 5min before measuring the glycogen content. Glycogen content was measured by colorimetric reaction using 25µL of prepared sample. 10µL of 80% aqueous phenol and 200µL of sulphuric acid were added to the samples on 96-well plates. Absorbance was measured using a multi- detection microplate reader (Synergy HT, BIO-TEK) at 490 nm. The concentration of glycogen in the samples was calculated based on a mussel glycogen standard (Sigma, Saint Louis, MO).

Total lipids were determined based on Blight and Dyer method

(Hanson and Olley, 1963). In this way, total lipids from a known weight of tissue was extracted and purified by homogenization in a suitable excess of chloroform/methanol/water (2:2:1.8 v/v/v). Following this, total lipids in chloroform solution were determined by weighting the eluate after removal of the solvent by rotary evaporation. All lipid extracts were then stored in chloroform at -30°C to await analysis.

Fatty acid methyl esters (FAME) were separated and quantified using an Agilent 7890 Gas Chromatographer equipped with auto-injector (Agilent Technologies, United States). The gas chromatograph is equipped with a Flame-ionization detector (290°C) and a DB-WAS 122-7032 Agilent Technologies capillary column (30m _ 0.25mm I.D., film 0.25μm). Helium was used as a carrier gas and initial oven temperature was 50°C, followed by an increase at a rate of 10°C·min^{-1} to a final temperature of 240°C for 9min. Individual FAME were identified by comparison of retention times with four commercial fatty acid standards (PUFA 1, PUFA 3, BAME and 37-component) supplied by Supelco (Bellefonte, PA) for area percent normalization. At each sample relative quantities were expressed as weight percent of total fatty acids.

Sensory evaluation/Consumer testing

Thirty regular domestic consumers of mussel were randomly recruited by email from AZTI's database. They were segmented by

gender (50% men) and age range (18-70 years). In order to remove debris and any remaining byssal thread, 150 mussels were washed and scrubbed for the sensory analysis. Samples were steamed at 100 ± 1 ºC for 1 minute and then served at 65 ± 1 ºC. Mussels were presented to consumers on white plastic plates labeled with 3-digit random numbers, containing 2 mussels from each origin (experimental from open ocean and commercial from Galician Rías). Consumers tasted one mussel from each origin. The consumers received oral and written instructions and were provided with water and unsalted-crisp bread for palate cleansing after each tasting. Each consumer tasted the samples in randomized order and evaluated 5 attributes (appearance, odour, taste, texture, and global acceptance) with a structured 9-point scale ranging from 1 (equivalent to I dislike it very much) to 9 (equivalent to I like it very much). The 9-point hedonic scale (Peryam and Girardot 1952; Peryam and Pilgrim 1957) is the most commonly used scale for testing consumer preference and acceptability of foods (Schutz and Cardelo 2001). This kind of scales produce central tendency effects on panelist, which reduces the options to a 7 point-scale (Villanueva *et al.* 2005). Despite this possible reduction, a 9 point-scale with an arithmetic adjustment can always be straightforward re-scaled to a 7 point scale (Epler *et al.* 1998). This enables the comparisons with different scales formats with

less response options (Dawes *et al.* 2008), which are common in current scientific literature. Consumers' purchase intention was also assessed with a structured 5-point scale ranging from 1 (equivalent to I won't buy it) to 5 (equivalent to I'll buy it). The same test was repeated three times with each panelist. Consumers tasting was performed in the sensory laboratory of AZTI- Tecnalia specifically designed according to ISO 8587:2006.

Statistical analysis

Relative shell morphology was investigated through the general linear function, where y and x are size-related measures and *a* and *b* are constants (i.e. intercept and slope; which represents the coefficient of relative shape) (Pauly 1983). Similarly, allometric equations were generated separately for mussels from each origin, using linear regression and logarithmic transformation. The differences between allometric coefficients (*b*) from each origin were evaluated using ANCOVA (Rohlf and Sokal 1981).

Means and standard deviations (mean ± SD) of data on morphometrics, proximate composition (humidity, ash, protein, glycogen, and lipid content), as well as fatty acid profiles, of mussels were plotted using Sigmaplot (11.0) statistical and graphical software (Systat software). Statistical analyses were carried out using a statistical

package (SPSS 20.0 for Windows®). Sensory attributes and evaluation data were analyzed using the R-project software (R Development Core Team, 2008). A signifcance level of 0.05 was used. Normality was tested by Shapiro-Wilk test and the homocedasticity was checked using Leven's test. The differences between mussel products type (experimental and commercial products) were compared using independent sample t-test. Arcsine transformation (Zar 1984) was applied to the data expressed as percentage.

RESULTS

Morphometry

The biometric results of the two mussel products (experimental open ocean and commercial from Galician Rías) tested within the present study are shown in Table 1. No statistical differences between the shell length (L) of both mussel products were detected. Conversely, significant differences were detected for the measurements of shell height (H) and shell width (W) between both mussel products. As observed, the open ocean mussel individuals from the present study displayed significantly higher morphometric values than those from the commercial samples.

The allometric relationships between shell length (mm) and shell width (mm), shell length and shell height (mm), or between shell width (mm) and shell height (mm) of both mussel products are shown in Table 2.

Table 1 M. *galloprovincialis*. Biometric characteristic of the mussel shell and proximate composition of mussel' tissue pools from experimental open ocean (SE the Bay of Biscay; Basque region) and sheltered Rías (SW Bay of Biscay; Galician region) conditions.

	Experimental	Commercial	One way-ANOVA	
	Open Ocean	Galician Rías	F	p-value
L (mm)	70.29 ± 7.96	69.65 ± 6.11	0.037	0.849
H (mm)	37.56 ± 4.59*	33.58 ± 2.48	10.471	0.003
W (mm)	24.58 ± 4.04*	21.18 ± 4.59	5.538	0.024
Moisture (%)	78.77 ± 1.54	76.57 ± 1.77	2.661	0.178
Protein (Dry Weight %)	49.52 ± 4.29	44.79 ± 4.02	1.962	0.234
Lipid (Dry Weight %)	6.27 ± 1.47	6.30 ± 1.34	0.001	0.976
Ash (Dry Weight %)	13.00 ± 1.16*	9.40 ± 0.82	19.253	0.012
Carbohydrates (Dry Weight %)	31.21 ± 4.87	39.51 ± 6.07	3.356	0.141

[1] Mean ± standard deviation, n = 20.

Table 2 *Mytilus galloprovincialis*. Parameter of shell shape (length (L), width (W) and height (H)) regression equations used to relate shell length (mm) to shell height (mm) or shell width (mm), and shell width (mm) to shell height (mm) of different mussel products (experimental vs. commercial); equation fitted to log10 X= log10 a + b log10 Y; where a intercept and b slope, and X and Y shell measurements. Error of correlation coefficient (r), number of specimens (n) used to derive equation are also given.

Allometric relation	*Mussel product*		*B*	*SE*	*t*	*sig*	*95% CI*		R^2	*N*
							Upper	*Lower*		
Log H/ Log L	*Experimental*	*Intercept*	-0.185	0.224	-0.823	0.421	-0.656	0.287	0.773	20
		Slope	0.952	0.122	7.834	0.000	0.697	1.208		
	Commercial	*Intercept*	0.390	0.241	1.620	0.123	-.116	0.897	0.552	20
		Slope	0.616	0.131	4.709	0.000	0.341	0.891		
Log W/ Log L	*Experimental*	*Intercept*	-0.874	0.284	-3.075	0.007	-1.471	-.277	0.882	20
		Slope	1.225	0.154	7.951	0.000	0.901	1.549		
	Commercial	*Intercept*	-0.501	0.243	-2.066	0.053	-1.011	0.008	0.873	20
		Slope	1.000	0.132	7.595	0.000	0.724	1.277		
Log H/ Log W	*Experimental*	*Intercept*	0.735	0.161	4.557	0.000	0.396	1.073	0.775	20
		Slope	0.604	0.116	5.197	0.000	0.360	0.848		
	Commercial	*Intercept*	0.790	0.149	5.287	0.000	0.476	1.104	0.757	20
		Slope	0.548	0.111	4.917	0.000	0.314	0.782		

Based on ANCOVA analysis (Table 3), the factor of product type significantly affected both the linear relation between log transformed shell length (mm) and log transformed shell height (mm), as well as the linear relation between log transformed shell length (mm) and log transformed shell width (mm). Higher values were found in the experimental mussels. Finally, no ANCOVA analysis was performed to compare the linear relation between log transformed shell height (mm) and log transformed shell width (mm) as significant interaction between the factor (product type) and the covariable (log transformed shell width (mm)) was observed. Hence, no direct relation between the change in shell width (mm) and the change in shell height (mm) among mussel products could be assessed.

Table 3 *Mytilus galloprovincialis*. Mussel product type effects on shell length (mm) to shell width (mm) and on shell length (mm) to shell height (mm), allometric relations analyses. Regression equation exponent b (slope) were compare using ANCOVA (Sokal and Rohlf, 198), with α set at 0.05.

Allometric relation	*Source of variation*	*ANCOVA*			R^2
		df	*F*	*sig*	
Log H / Log L	*Log Length (covariable)*	1	83.561	0.000	
	Product type (factor)	1	29.992	0.000	0.759
	Error	37			
Log W/ Log L	*Log Length (covariable)*	1	121.922	0.000	
	Product type (factor)	1	19.953	0.000	0.803
	Error	37			

Biochemical composition & Fatty acids

The proximate composition of both mussel products are shown in Table 1. Moisture levels were relatively high within both samples. Mussels from commercial origin (Galician Rías) showed slightly higher moisture values (78.77% vs. 76.57%) than mussels from the open ocean conditions of the present study, yet significant differences were not detected.

Significantly lower ash levels were detected within the commercial individuals (13.00% vs. 9.40%) compared to the study ones.

Lipid contents were slightly higher in the commercial samples (6.27% vs. 6.30%), although no significant differences were found. Protein contents did not show any significant differences between mussel products. Finally, glycogen levels (71.06 ± 16.71 $mg \cdot g^{-1}$ and 100.59 ± 25.05 $mg \cdot g^{-1}$ at experimental and commercial mussels, respectively) were slightly higher in commercial samples, although differences were not statistically significant.

The FA composition of both mussel products are shown in Table 4 and 5. The saturated fatty acids (SAFA) accounted for 29% in experimental mussels and significantly lower content (25%) for commercial mussels. Saturated fatty acid predominated over the monounsaturated fatty acid (MUFA) with 12% for the experimental and significantly higher level

(22%) for the commercial samples. Both mussel products showed the majority of fatty acids consisted of polyunsaturated fatty acids (PUFA), with significantly higher level (59%) for experimental mussels than for commercial mussels (52%).

In the case of SAFA, palmitic acid (16:0) was predominant (18.91% and 15.50%) in both products, followed by stearic acid 18:0 in the open ocean mussels (4.70%), and by myristic acid 14:0 in the commercial mussels (4.14%). Significant differences (One-Way ANOVA; $P<0.05$) in the SAFA profile (i.e. the sum of pentadecanoic 15:0; palmitic 16:0 and behenic 22:0 acids) were found between both mussel products.

Regarding MUFA, the commercial mussel from Rías displayed abundant related palmitoleic acid 16:1ω7 (11.4%). This was followed by vaccenic acid 18:1ω7 (3.19%) and eicosenoic acid 20:1ω7 (2.03%). Conversely, the observed trend of MUFAs in the open ocean experimental individuals was not equivalent; the major acid was gondoic acid 20:1ω9 with 3.48%, followed by palmitoleic acid 16:1ω7 with 2.40% and oleic acid 18:1ω9 with 2.20%. Thus, significant differences (One-Way ANOVA; $P<0.05$) were found between MUFA of both mussel products.

Regarding PUFAs, eicosapentaenoic (EPA) (20:5ω3) and docohexaenoic (DHA) (22:6ω3) acids were the most important.

Commercial mussels showed higher EPA (20:5ω3) with 24.71% and less DHA (22:6ω3) with 5.66%, whereas the experimental open ocean mussels showed less EPA (20:5ω3) content with 9.85% and higher (DHA (22:6ω3) with 23.13%. Thus, the DHA/EPA ratios were significantly different (One-Way ANOVA; $P<0.05$) between mussel products.

Nonmethylene-interrupted dienoic fatty acid (i.e. 22:2 NMIDa and 22:2 NMIDb) were higher in the commercial samples (4.66%) than in the experimental ones (1.95%). Differences at NMID level were statistically significant (One-Way ANOVA; $P<0.05$) between mussel products.

Finally, terrestrial fatty acids (i.e. 18:2ω6 + 18:3 ω3) accounted for the 3.85% in experimental mussels and 1.64% in the commercial ones; differences between mussel products were statistically significant (One-Way ANOVA; $P<0.05$).

Table 4 M. *galloprovincialis*. Fatty acid compositions (percentage of total fatty acids) of mussel' tissue pools analyzed during the comparative study between the experimental open ocean mussels from the SE Bay of Biscay and commercial mussels obtained from the sheltered Rías from Galicia..

Fatty acids	Experimental Open Ocean	Commercial Galician Rías
14:0	2.52 ± 0.80*	4.14 ± 0.60
15:0	0.57 ± 0.07 *	0.36 ± 0.02
16:0	18.91 ± 1.29 *	15.50 ± 0.43
17:0	1.90 ± 0.32*	1.12 ± 0.20
18:0	4.70 ± 0.93	3.74 ± 0.52
20:0	0.06 ± 0.05	0.02 ± 0.04
22:0	0.03 ± 0.03	0.18 ± 0.03 *
ΣSAFA	28.69*	25.05
14:1	0.42 ± 0.07	0.99 ± 0.17 *
16:1ω7	2.40 ± 0.87	11.14 ± 1.42 *
16:1ω5	0.30 ± 0.05	0.25 ± 0.02
17:1	0.19 ± 0.16	0.40 ± 0.10
18:1ω9	2.22 ± 0.68	1.14 ± 0.05
18:1ω7	1.22 ± 0.15	3.19 ± 0.07 *
20:1 ω11	1.21 ± 0.23	1.32 ± 0.17
20:1ω9	3.48 ± 0.28 *	1.59 ± 0.19
20:1ω7	0.47 ± 0.05	2.03 ± 0.16 *
22:1	0.06 ± 0.06	0.24 ± 0.14
ΣMUFA	11.98	22.28 *
16:4ω3	8.32 ± 2.25	3.04 ± 4.70
18:2ω6	2.03 ± 0.32*	0.87 ± 0.02
18:3ω3	1.82 ± 0.44*	0.77 ± 0.09
18:4ω3	3.74 ± 1.23*	1.35 ± 0.24
20:2 α	2.76 ± 0.36*	1.57 ± 0.31
20:2 β	0.25 ± 0.04	1.14 ± 0.35*
20:2ω6	0.71 ± 0.12*	0.38 ± 0.03
20:4ω6	1.30 ± 0.21	2.61 ± 0.12 *
20:4ω3	0.34 ± 0.13	0.64 ± 0.23
20:5ω3	9.85 ± 1.06	24.71 ± 2.21 *
22:2 NMIDa	0.73 ± 0.10*	0.12 ± 0.10
22:2 NMIDb	1.22 ± 0.13	4.54 ± 0.43 *
21:5ω3	1.20 ± 0.16	1.09 ± 0.06
22:4ω6	0.20 ± 0.04	1.90 ± 1.54
22:5ω6	0.61 ± 0.03*	0.15 ± 0.15
22:5ω3	1.12 ± 0.11	2.13 ± 0.21 *
22:6ω3	23.13 ± 2.20 *	5.66 ± 0.47
ΣPUFA	59.33 *	52.67
NMID	1.95	4.66 *
Terrestrial (18:2ω6 + 18:3 ω3)	3.85*	1.64
Ω3/Ω6	10.21	6.67
DHA/ EPA	2.35 *	0.23

[1]Values (mean ± SD, n=10)

Sensory evaluation/Consumer testing

The panelist male:female ratio (%) participating in the present study was 37.7:63.3. The range of ages between participants was distributed as follows: 18-30 years old (13.3%); 31-40 years old (40%); 41-50 years old (33%) and 51-60 years old (13.3%). The 77% of participants were regular mussel consumers (3.3% more than once a week; 26.7% once a week; and 46.7% once a month), whereas the 23.3% declared to be occasional mussel consumers.

The resulted acceptance for both mussel products was around 7 points out of 9 (Fig 2). No significant differences (*t-test*; $P>0.05$) were detected for any of the sensory attributes analyzed between both products (Table 5). The results of the qualitative assessment showed that both mussel products showed good appearance and odour. The results of the qualitative assessment showed that both mussel products showed good appearance and odour. Particularly, the experimental ones showed to provide more intense sea-flavor with a slightly softer and more juicy texture. Some other aspects such as the whitish colour on the muscle tissue and/or the salty taste were criticized when observed. A large proportion of the panelists (70%) expressed potential interest in purchasing the experimental product, while a small minority (17%) did not. The remaining 13% of panelists did not express a purchase

preference. For the commercial mussels, 83% of the panelists showed purchase intention, while 10% showed uncertainty (or doubt) and 7% would not buy it (Fig 2).

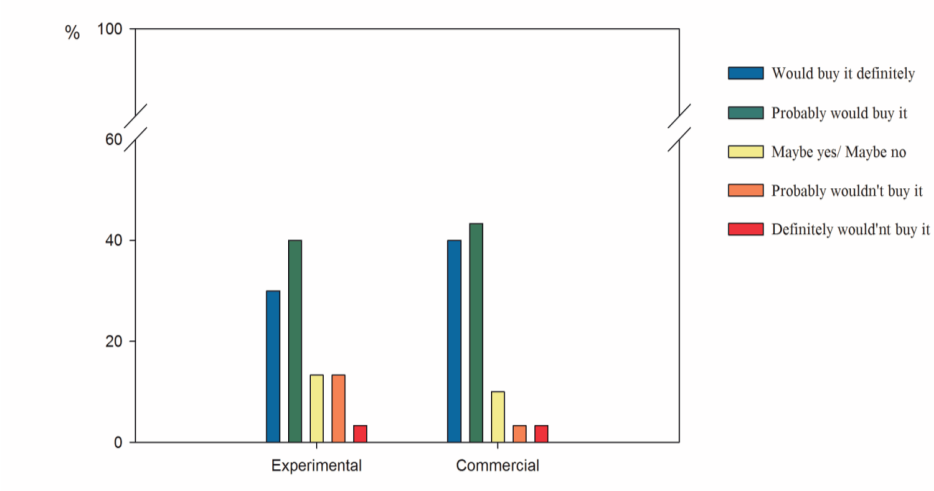

Figure 2 *M. galloprovincialis. Buying-intention results carried out on the comparison between mussels from the open ocean in the SE Bay of Biscay ("experimental") and from sheltered estuaries in the SW Bay of Biscay ("commercial").*.

Table 5 M. *galloprovincialis*. T-student analysis carried out for all sensorial attributes analyzed in experimental mussel from the open ocean conditions of the SE Bay of Biscay and mussels cultured in at sheltered Rías from Galicia.

	Experimental	Commercial	*t*-student	
	Open Ocean	Galician Rías	t	P-value
Appearance	6.467 ± 2.097	7.033 ± 1.066	1.32	0.194
Odour	6.967 ± 1.402	7.3 ± 1.088	1.029	0.308
Flavor	7.533 ± 1.106	7.4 ± 1.303	-0.427	0.671
Texture	7.033 ± 1.586	7.533 ± 0.973	1.472	0.148
Global impression	6.9 ± 1.517	7.333 ± 1.269	1.2	0.235

[1]Values (mean ± SD, n=30)

[2]*Statistical significance set up at ($P < 0.05$) (pairwise comparison).

DISCUSSION

All mussels consumed in the Basque region, during recent decades, are imported (Gracia 1996). Currently, the only way to cultivate mussels in the Basque Country is offshore and with submerged longline method (Azpeitia *et al.* 2016; Chapter IV). The aim of the study was to compare if experimental open ocean cultured mussels of commercial size were as competitive as local market leader of the same commercial size. No significant differences were observed in shell length between both products, which validated the analysis of quality differences among mussel products as well as the analysis of consumer perception among products. Although panelist were not able to inspect mussel shells, only mussels without visible damage (open valves or broken shell) were used in the study. Direct influences on the appearance of the product should be derived from the differences found at biometric levels between both mussel (M. *galloprovincialis* Lmk.) origins. Different shell shapes among mussel product were observed. Mussels cultured in Galician Rías were found to be thinner (reduced W . L^{-1} ratio) and lower (reduced H . L^{-1} ratio) than experimental open ocean mussels. Commercial mussels may have been cultured at higher densities than experimental mussels. Stocking density on experimental mussels was 681 individuals/m, whereas commercial label does not provide the consumer with those

culture characteristics. Nevertheless, literature value for standard mussel density on ropes cultured in sheltered Galician Rías is between 700 -1000 mussels·m^{-1} (Irisarri *et al.,* 2014a). Cubillo *et al.* (2012a) observed that mussels cultured at higher densities displayed a lower shell height to shell length ratio compared to mussels cultured at a lower density. Similarly, the author observed that mussels cultured at higher density displayed lower shell width to shell length ratio compared to those cultured at low densities.

Regarding differences in oceanographic conditions, mean current speed in the experimental site was significantly higher than values reported from previous studies carried out in Galician sheltered Rías (Pérez-Camacho *et al.* 2014). Similarly, highly significant wave height scenarios were also found (with peaks of 7,96m) at the experimental site. Indeed, only open ocean mussel growth studies of Buck and Buchholz (2004) and Langan (2000), from the German Bight and the Gulf of Maine, respectively, showed similar wave height scenarios (with peaks of 7.96 m). The mussel shells of the experimental site were characteristically round, wide and great in thickness. Although these observations were not validated through specific comparisons, they are consistent with the findings from previous studies developed with mussels from wave beaten shores (Akester and Martel, 2000, Lewis and Powel, 1961, Seed

1968).

Despite the limited information available on the culture method and culture characteristic of commercial mussel product, the new product cultured at the experimental site would compete with current commercial products. No differences regarding mussel appearance of cooked samples were pointed out by the group of local panelists during the sensory test.

In this study, no relevant differences at biochemical composition level other than ash content were observed between the open ocean experimentally cultured mussels and the commercially purchased mussels from Galician Rías. Biochemical composition of mussels is affected by water temperature, nutrient availability, and the reproductive cycle of individuals (Fernández-Reiriz *et al.* 2007). These multiple factors change seasonally, which, in turn, cause seasonal changes in biochemical composition of mussels (De Zwaan and Zandee 1972; Dare and Edwards 1975; Ruano *et al.* 2012). As reported by previous authors, major differences in biochemical composition relied on: (i) feeding behaviour, in terms of filtration capacity and clearance rate (Vilela 1950; Heral *et al.* 1980); (ii) quality and quantity of seston available in the environment; and (iii) state of sexual maturity of individuals (Fernández-Reiriz *et al.* 2007).

This study showed similarities on proximate composition between both mussel products, consistent with previous findings from mussels from Galician waters as reported by Fuentes *et al.* (2009). This may be explained by similarities occurring within the main factors regulating growth and biochemical performance in both culture conditions. Both culture conditions have a close annual average in environmental conditions, the same seasonality in reproductive cycles, and/or the same genetic heritage due to geographic proximity. However, open ocean mussels exhibited a higher amount of ash when compared to commercial ones from Galician Rías. Fuentes *et al.* (2009) observed similar values of ash content in commercial product from Galician Rías when comparing mussel product from different Spanish regions. Higher ash content than open ocean mussels of this study were also observed in the same study in mussels from Ebro Delta and from Valencia (NE and SE Spain, respectively). Mussels cultured in the exposed environmental conditions (e.g. phytoplankton variability, strong hydrodynamics, and very marked seasonal changes in seston composition) may experience situations of stress or may have greater energetic expenditure (Azpeitia *et al.* 2016; Chapter IV) than mussels cultured in sheltered conditions. Oliveira *et al.* (2015) found similar results when comparing open ocean mussels to sheltered cultured mussels. The authors related this

difference to the hydrodynamic conditions found by mussels in the offshore culture areas, which would interfere with mussel metabolism in a set of complex interactions between temperature, food availability, growth, and reproductive cycle (Gabbot 1976). Thus, ash content is influenced by many abiotic factors such as salinity and presence of metals on the water column (Okumuş and Stirling 1998), as well as by the nutritional state and the reproductive cycle of the animal (Beninger and Lucas 1984). Regarding nutritional state, Mayzaud (1979) associated an increase in ash content of zooplankton with a state of starvation, and Wilkins (1967) observed the same in herrings. Nevertheless, this higher ash percentage may account for the decrease in other constituents (e.g. lipids, proteins) rather than an increase in the amount of ash, as data analysis was performed with relative percentages and not in absolute terms. Despite these differences in ash content, similar general properties at biochemical level should be expected when comparing the open ocean mussels from the SE Bay of Biscay to their commercial counterparts from another northern Spanish region.

Depending on the time of the year, levels of nutrients, both in Galician Rías and in the open ocean of the SE Bay of Biscay, differ. In Galician Rías, upwelling events typically take place during spring-summer. Highest chlorophyll levels have been recorded during spring

(<5 $\mu g \cdot l^{-1}$)(Irisarri *et al.* 2014a). However, upwelling events are usually followed by thermal stratification periods during the summer which might result in a significant reduction of chlorophyll levels up to 0.4 $\mu g \cdot l^{-1}$ (Irisarri *et al.* 2014a). The thermocline layer prevents nutrients from fertilizing the surface and chl-a eventually becomes depleted. The author corroborated this with a negative scope for growth (SFG) observed in mussel, when abnormally high temperatures are coupled with aforementioned low energy intake in Galician Rías in summer. In the SE Bay of Biscay, there is also a thermal stratification during summer, which decreases phytoplankton biomass to lower values than those described in Galician Rías (0.2 μg·l–1 in surface waters) (Morán *et al.* 2012). However, during summer, short periods of atmospheric instability (storms) may act as modulation factors of this bloom decay in the SE Bay of Biscay. Although inputs of nutrient-rich continental waters can be observed, the intensified aforementioned current velocities may also resuspend inorganic sediments. Thus, in storm conditions, a dilution of available particulate organic matter (POM) for bivalve filter-feeders can be expected (Cranford *et al.* 2011; Irisarri *et al.* 2013; Zuñiga *et al.* 2014) in the experimental site.

The biochemical composition of the present contribution are consistent with previous studies reporting that mussels are high in

proteins and low in calories and fat (Shahidi 2004; Fuentes *et al.* 2009; Gallardi *et al.* 2014). Indeed, some authors have reported that a daily protein intake of 0.75 g·kg body weight (which means that with 100g of mussels for a person of 70kg) is recommended to satisfy approximately 23% of the daily protein requirements (Garcia-Gabarra 2006; Stankovic *et al.* 2011; James 2013); thus, demonstrating the rich nutritional value of mussels for human consumption.

In addition, it is also generally accepted that mussels represent an important source of essential fatty acids and particularly for long-chain polyunsaturated variety (PUFAs) (James 2013). In this study, not only concentration levels but also their representativeness on the profile of the major fatty acid groups (PUFA, MUFA and SAFA) are consistent with previous mussel studies (King *et al.* 1990; Freites *et al.* 2002b; Orban *et al.* 2002). Irisarri *et al.* (2014b), observed a selective accumulation of PUFA in different tissues of mussels. Mussels have a limited capability for de novo synthesis of PUFAs (Alkalani *et al.* 2007) and acquire them from diet. The unsaturation degree of the FA increases during the colder seasons, as mussels require these FA to maintain membrane fluidity (Hall *et al.* 2002). The contrary is observed in warmer seasons and thus, the PUFA level varies in opposite fashion to SAFA level. Mean seawater temperature in open ocean experimental site in summer was

slightly higher (Azpeitia *et al.* 2016; Chapter IV) than the typical summer temperature in Galician Rías (Pazos *et al.* 1997), which could have promoted a faster decrease in the unsaturation degree. This may have caused SAFA level to be higher in experimental mussel than commercial ones. Several authors have reported that higher proportions of SAFA may represent bacterial loads or organic-detritus rich environments (Galap *et al.* 1999; Freites *et al.* 2002b). However, open ocean conditions are related to high hydrodynamic scenarios which favour marine water recycling and consequently less detritus accumulation compared to sheltered sites. Despite this higher water recycling, resuspension of material may have occurred at the experimental site. Moreover, regarding bacterial biomarker FA (15:0, 17:0, 18:1ω7 and 18:1ω7/18:1ω9>1; Budge *et al.* 2001), higher values were observed in mussels from Galician Rías compared to experimental mussels. This may indeed support the aforementioned notion that temperature differences may well lead to higher SAFA levels in experimental mussels.

Major fatty acid levels in marine bivalves are generally known for being highly dependent on biochemical and environmental conditions (Lucas and Beninger 1985; De Moreno *et al.* 1980; Fernández-Reiriz *et al.* 1989; Fuentes *et al.* 2009). Nevertheless, the aforementioned results are consistent with current existing knowledge on mussel biochemistry

and could be of use in further research in the same field at a local level.

Palmitic acid (16:0) has been reported in numerous studies (King *et al.* 1990; Freites *et al.* 2002a; Orban *et al.* 2002; Alkalani *et al.* 2007; Fuentes *et al.* 2009) as the major saturated fatty acid (SAFA) present in mussels. The experimental open ocean mussels herein displayed not only the same trend, but also, consumers reported a more intense sea-flavor after tasting the product. This would be consistent with Oliveira *et al.* (2015), who found that palmitic acid along with DHA are mainly responsible for mussel' flavour.

PUFA levels of both mussel products herein were abundant and consistent with results from previous mussel studies from other localities (King *et al.* 1990; Orban *et al.* 2002; Freites *et al.* 2002b; Dridi *et al.* 2007; Alkalani *et al.* 2007; Fuentes *et al.* 2009), where healthy mussels always contain PUFA levels near to 50% of the total fatty acids proportion. As reported by Musa-Veloso *et al.* (2011) a dose of 250 $mg \cdot day^{-1}$ of the long-chain omega-3 fatty acids EPA and DHA (PUFA) is strongly recommended for human health. In this way, although mussels are low in lipids, these lipids are of high quality and provide a healthy source of omega-3 fatty acids with numerous nutritional benefits (Shahidi 2004; Byrd-Bredbenner *et al.* 2009; James 2013). A high ω6/ω3 ratio has also been reported as harmful to human health (i.e. over values of 12/50;

Ackman 1990; Shahidi and Miraliakbari 2004; Fuentes *et al.* 2009). The results on the EPA and DHA of the present study were suitable within all ranges for both types of mussels examined.

Suspension feeding bivalves feed on living and non-living available material in their habitat (i.e. generalist filter feeders) (Ward and Shumway, 2004). They select particles from seston, separating the grain from the chaff, and acquire the optimum energetic budget (Ward and Shumway, 2004). Seston consists of plankton of a wide range of sizes and palatability, material resuspended from the benthos, agregates, detritus, fecal pellets as well as microorganisms (Ward and Shumway, 2004, Karayücel *et al.* 2010). Fatty Acids (FA) have been succesfully used as trophic markers of bivalves to identify food sources, habitat preferences, feeding strategies and trophic links (Kharlamenko *et al.* 1995, 2001; Prato *et al.* 2010). Several studies on mussel *Mytilus* galloprovincilais Lmk. (Freites *et al.* 2002a b; Ezgeta-Balić *et al.* 2012; Irisarri *et al.* 2014a) found that variability in nutritional quality of seston was explained by the variance of FAs found in mussels. Bivalves have some capability to synthesise FAs independently of diet (Ventrella *et al.* 2013), but they are not capable of biosynthesizing *de novo* polyunsatured FAs (PUFAs) (Fernández-Reiriz *et al.* 2011). In addition, fatty acids transferred from primary producers, when incorporated by bivalves, do not undergo

major changes (Graeve *et al.* 1994; Xu and Yang, 2007), and thus, fatty acid measurments are a qualitative measurement of both energy transfer and assimilation of nutrients in mussel tissue. FA signature in mussel tissues can reveal the food source of the bivalve (i.e, several weeks prior) (Ezgeta-Balić *et al.* 2012) and thus, certain FA and their ratios can be used as biomarkers of the diet of the mussel. With these FA ratios, the seasonal contribution of bacteria, phytoplankton classes, as well as microzooplankton to the diet of the mussel can be assessed (Budge *et al.* 2001; Handå *et al.* 2012). Such analysis is an important factor in understanding the feeding physiology of mussels (Xu and Yang 2007).

In spite of presenting healthly EPA and DHA proportions, the experimental open ocean mussels of the present study showed less EPA and higher DHA contents than the commercial ones from Galician Rías. EPA level could be related to the ingestion of dinoflagellates (Freites *et al.* 2002a), whereas DHA could be a consequence of consumption and accumulation of diatoms (Handå *et al.* 2012). Sidari *et al.* (1998) observed that *Mytilus galloprovincialis* Lmk. as a preference selected dinoflagellates rather than diatoms. DHA/EPA ratio was >1 in experimental mussels, whereas, it was <1 in commercial mussels. These FA trophic markers (Budge *et al.* 2001; Dalsgaard *et al.* 2003) suggest mussels from the experimental site feed on dinoflagellates.

Additionally, the ratio 16:1ω7/16:0 was <1 in experimental mussels and >1 in commercial mussels. Thus, all these FA trophic markers suggest commercial mussels feed on diatoms rather than dinoflagellates (Budge *et al.* 2001; Dalsgaard *et al.* 2003). No water samples were collected for phytoplankton analysis. However, as described by Álvarez *et al.* (2008) in the western Iberian Peninsula (i.e. Portugal and Galicia) during spring-summer the continental shelves are strongly affected by upwelling pulses. These natural events are caused by the prevailing winds and the coastline orientation, causing an inflow of deep nutrient-rich waters which boosts phytoplankton productivity (Varela *et al.* 2005). Moreover, as described by Figueras *et al.* (2002) and Álvarez-Salgado *et al.* (2008), these upwelling pulses result in the transportation of dinoflagellates to the continental shelf and leave the embayment dominated by diatoms. Similarly, Irisarri *et al.* (2014b) found that diatom FA biomarkers were significantly higher in mussel tissues from Galician Rías during spring-summer although those FA trophic markers were >1 throughout the entire year. Oliveira *et al.* (2015) also reported higher levels of EPA in mussels (*M. galloprovincialis* Lmk.) from the Galician Rías compared to individuals of the same species but different culture origin (S Atlantic Sea (Portugal)). The authors suggested that the resultant DHA/EPA ratios would reflect the higher relative ingestion of diatoms. The contribution,

however, of phytoplankton (as diatoms and dinoflagellates) to the diet (derived by 16:1ω7/16:0 and 20:5ω3/22:6ω3 ratios) was proportionally low and moderate in open ocean and commercial (M. *galloprovincialis* Lmk.) mussels, respectively. In the present study mussels were harvested in July along the north Spanish coast. There is a high likelihood that previous poor environmental conditions (Prato *et al.* 2010) or post-spawning stages (Garmendia *et al.* 2010; Ortiz-Zarragoitia *et al.* 2011; Múgica *et al.* 2015; Cuevas *et al.* 2015) may have affected mussels' fatty acids composition in summer.

Fatty acid trophic markers of terrestrial organic sources (i.e. (18:2ω6 + 18:3ω3)) higher than 2.5 were described by Budge and Parrish (1998b) as indicative of a terrestrial organic diet source (i.e. terrestrial plants). A significant input of terrestrial markers was observed in the present study, where the experimental open ocean mussels showed to exceed the threshold. This may be related to the aforementioned differences in seston dynamics in Galician Rías and in the SE Bay of Biscay during summer season.

Finally, the presence of 22:2NMID acid was lower in experimental mussels compared to commercial ones. Sánchez-Lazo and Martínez-Pita (2012) also reported the presence of this fatty acid, although accounting for very little proportion.

The sensory analysis herein was consistent with previous mussel studies (Kryzynowek and Wiggin 1979; Ablett *et al.* 1986; Gökoglu 2002). The panelists were able to determine the mussel product qualitative characteristics on appearance, odour, flavor, texture, and global perception. Both types of mussel (open ocean and commercial) reached scores of 7 over 9-points at hedonic scale for all sensory attributes. In general terms, even though no triangle test was done, the sensory analysis session was performed stable and consistent among panelists.

Regarding appearance and odour, both characteristics were scored slightly lower for the open-ocean samples when compared to the commercial ones from Galician Rías. Observed discrepancies on mussel appearance could be related to inherent biological and/or operational factors (e.g. higher variability in the coloration of mussel meat from open ocean conditions; previous industrial procedures applied on the commercial samples, etc.). The results of the present study showed no significant differences in tasting levels between open ocean and commercial products. However, most panelists also identified some distinctive attributes such as, intense sea-flavour, softer and more juicy textures, and salty tastes in the open ocean samples.

These results may specifically be related to the absence of previous depuration requirement on the open ocean samples as a result of

having an origin with a class A production area according to EU and local public legislation. During depuration processes, mussels are fasted and animals excrete their metabolic waste products, which include salts (Lee *et al.* 2008). Despite depuration of commercial mussels, the organoleptic characteristics did not differ greatly between both mussel products. The results on texture, at the time of the sensory analysis, also revealed softer meat in experimental mussels. Nevertheless, differences were not statistically significant and thus, it can be concluded that softer texture does not imply mussels are less desirable. It is noteworthy that desired quality among fishery products relies on regional preferences, consumers' attitudes and methods of preservation and/or consumption (Haard 1992). Both mussel products reached 7 points out of 9 in qualitative category; a positive result considering the potential of open ocean mussels of the present study to be marketable in the near future. The tested consumers also expressed an intention to purchase both products (>70%). In general terms, this prescreening hedonic analysis showed that experimental open ocean mussels were similar in attributes to those routinely imported from the Galician Rías to the Basque Country. From a marketing perspective, the mussels cultured in the open ocean of the SE Bay of Biscay may be well accepted by consumers and fulfill the minimum requirements to fit and compete in the existing local

market. The results of the present study provide baseline information to validate nutritional value characteristics and consumers' acceptance of mussels (M. *galloprovincialis* Lmk.) cultured in the open ocean of the Bay of Biscay. In addition, as a commercial validation was positive, it is now worth investigating how changes in the culture method can optimize production and commercial activity of future aquaculture in the Basque Country.

Conclusion

Space available in coastal areas for aquaculture facilities is growing more and more limited and is subjected to strict jurisdictions and limited leases. In addition, the geography of the coast in many regions might not be sheltered enough for mooring rafts or longlines, while limited seston supply and/or polluted waters can also represent limiting factors for mussel coastal aquaculture. Hence, this study investigates an interesting alternative to traditional suspended mussel culture in coastal inlets.

This study is the first sensory assessment of mussels produced in open ocean from the SE Bay of Biscay. Firstly, this new information will be valuable for the development of strategies that favor origin specifications linked to health benefits which, in turn, would contribute to consumers ´decision-making processes (i.e. purchase intention and preferences). Secondly, it will help to predict cost-effectiveness during

mussel production and commercialization.

The approach of this study aimed to validate consumer acceptance as a meaningful factor in market feasibility studies. In addition, it also aimed to investigate if mussels cultured in the open ocean could compete with other mussels currently available on the market. However, future market needs will ultimately decide the final viability of mussel open ocean aquaculture.

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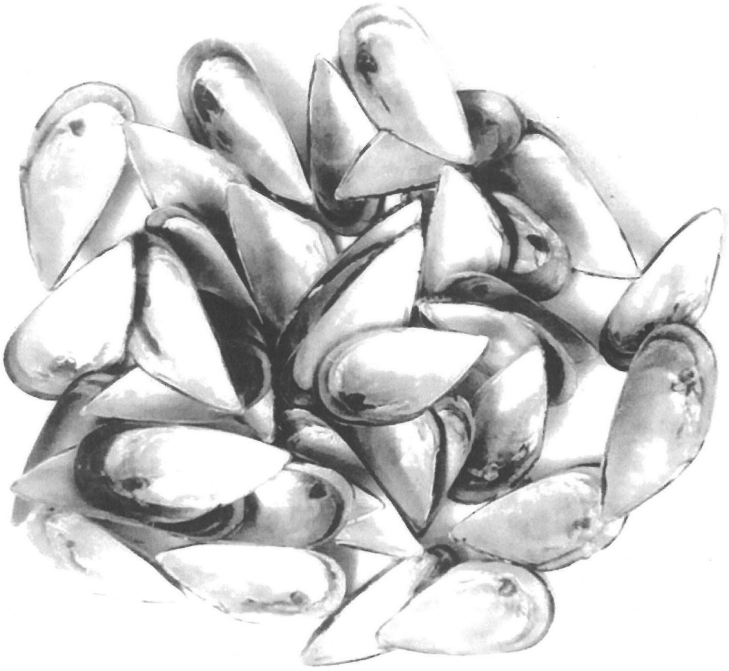

CHAPTER 7

CONCLUSIONS

1. The reproductive health status of *M. galloprovincialis* Lmk. from five sites, three estuarine and two nearshore open coastal waters, along the Basque coast were positively validated analyzing the sex ratios, developmental stages of gonads, as well as reserve tissue cycles of these populations.

2. For the first time, the annual settlement and recruitment patterns of *Mytilus galloprovincialis* Lmk. were assessed using collector ropes deployed at the aforementioned five sites along the Basque coast, near mussel beds. This simultaneous study enabled the discovery of a potential relationship between observed primary settlement and the spawning period of wild mussel populations and evidenced that recruitment was more determined by post-settlement processes than initial settlement abundances. Nevertheless, this study validated that mussel seeds collected at these sites, could serve to supply grow-out sites in the open ocean.

3. For the first time mussels aquaculture in a pilot system in the open waters of the SE Bay of Biscay, at two culture depth scenarios was validated. Growth as well as the biological performance of all mussels cultured on ropes was assessed seasonally and no significant effect of culture depth on mussel quality was

observed.

4. The study was able to separate the number of natural overset of mussels seeds on ropes form the original seeded batch. This enable the assessment of the real growth rate of the seeded batch. Year-round harvesting possibility was validated with the clear seasonal growth curves observed with von Bertanlanffy growth function. In addition, culture time variations depending on seeding time, were predicted.

5. Successful production rates were measured at study sites. Culture production loss (fall-off as well as mortality) rates at the study site were similar to values found at sites were mussels are successfully produced.

6. The number and quality of "small" (50-70mm) and "large" (>70mm) commercial size mussels harvested at the pilot system at both culture depth scenarios was assessed.

7. For the first time the food source of mussels cultured at the experimental site were assessed through fatty acid trophic markers analysis. Mussel feeding strategy was assessed at both culture depth, seasonally. Similarly, the comparison of feeding strategies between different commercial mussel products ("small"

vs. "large", as well as "experimental" vs. "commercial ones from Galician Rías") was assessed.

8. For the first time a sensory analysis session coupled with a comparative assessment of quality characteristics of mussels of commercial size was performed to test consumers acceptance to experimental mussels compared to routinely imported mussels of same commercial size from Galician Rías. The study validated that mussels cultured in the open ocean of the SE Bay of Biscay may well be accepted by consumers and may well fulfill the minimum requirements to fit and compete in the existing local market.

9. The finding of this work support mollusk aquaculture moving off the coast at the Basque Country. The observed biological performance and environmental data may well be used for culture management decisions.

FURTHER RESEARCH

This was the first pilot project for offshore mussel production in the Basque Country, and although a substantial amount of information was obtained, it may well be that the results are not sufficient for a complete evaluation of the bio-technical aspects of mussel culture. This research, however, has revealed some unexpected problems which could be managed by the time the product becomes commercially available. Nevertheless, results appeared to be conclusive for tested hypotheses.

Further research based on this thesis could help to gain an insight into the operational conditions to be encountered in the future at the proposed site.

The developed experimental design allowed for sampling of spat collector ropes as well as pairovet water column samples in the offshore system. Notwithstanding, time did not allow for analyses of these samples. However, understanding settlement in the offshore system could certainly help gain insights for mussel culture suitability (i.e., profitable spat recruitment) in the Basque Country.

Regarding technical aspects, an in-depth field study could be carried out to find the optimal length of rope, for maximum production. Ropes support mussel growth and biofouling weight. With deployment time, increased weight increases the risk of loss and mortality. Moreover,

the results of this thesis show that mussel production losses were considerable and thus, it is thought that shorter lengths (i.e., different from 12m-length ropes of the current raft-system) may enhance production yields per meter. Nevertheless, this shorter length would still need to provide reasonable production returns. Hence, different rope lengths could be tested evaluating mussel size frequency distribution, growth rate as well as yield. In addition, with rope of different lengths thinning out process implementation could also be analyze. Similarly, distance between ropes (i.e., 0.5 m) could be tested or even different seeding density. These could help to test differences on growth and survival. By all means(regardless of analysis), it is highly recommended to include mussel dislodgement force as a variable in such production analyses, as the pilot area is located in a highly energetic environment.

It would be valuable to study more than one complete growth period with different seeding times, as this would help to better understand the performance of mussels in the open water of Bay of Biscay predicted in this work. Subsequently, with this new biological and technical data, the management of year-long production could be implanted and managed.

In winter time of this study, there was a substantial loss of production coupled with a slight decrease in mussel quality. Moreover, winter was found to be the slow growing season. The acquisition of precise

information on every aspect of the environment, through water column monitoring, recording current speed and direction, temperature and salinity, oxygen levels, chlorophyll-a and suspended particulate matter, could help to understand and avoid unfavorable environmental factors typical of winter time. Currently AZTI Tecnalia manage two wave-buoys operating on the Bay of Biscay. In addition to these buoys, the submerged aquaculture pilot system has superficial buoys, which possess highly specialized remote control and monitoring capabilities. These are operated via leading-edge telemetry systems. It would be of future benefit to relate this information (partly used in this thesis) to interpret the newly found biological data. Similarly, the intensity and directions of the current regime and wave climate could be an important factor regulating larval dispersal. This kind of data could be used to address the settlement potential on offshore platforms, origin of newly settled larvae or where larvae that are produced in the pilot system disperse. The understanding of mussel larvae distribution in the water body on our coast and in the open water could help to attain successful settlement of mussels for successful aquaculture production.

It would be interesting to develop a operating methodology for the pilot culture system. For instance, it could help develop different culture strategies such as the possibility of using deeper water bodies for

seasonal depth management (i.e., in severe storms events ropes could be deepened).

The energy flow and carrying capacity of the ecosystem should be assessed and further investigated if the industry is to expand. Thereafter, it would be of use to have close cooperation with specialized manufactures for the development of prototypes, on-roots test and marketing. Further, knowledge and resources (i.e., captured lessons to be learned) should be transferred to solve common problems with a created common language to educate regulators and the public. In this way, the development of the coast will be fostered and an Integrated Coastal Zone Management (ICZM) as well as sustainable offshore aquaculture production could be attained. In doing so, the reproductive cycle, condition and morphology of mussels and larvae inhabiting the Basque coast should continue to be studied in order to assess future genotypic differences between wild populations. Such assessment would enable comparisons to be made so as to contribute to effective management.

Finally, future regulatory guidelines and legislation should determine which biological, technological and environmental criteria, following the code of conduct for responsible fisheries, will operate in the production of seafood in the open water of the Basque Country.

THESIS

During this work, several samples of wild mussel populations along the Basque coast were collected. The reproductive cycle and settlement pattern were assessed. Similarly, several samples of cultured mussels in the open ocean experimental site at two culture depth scenarios were collected. The biological performance and a preliminary economic viability were analyzed by different approaches. Overall, this work contributed to the knowledge of mussel performance in the region and further, validated that the baseline biological conditions for a successful mussel aquaculture industry are met in the case of the Basque Country.

ANNEX I

Author and year of publication	Species	Factor studied	Geographical region
Andreu, 1958	*M. galloprovincialis*	Biology, parasitology, growth and production	N.W. Spain
Bayne, 1965	*M. edulis*	Growth and metamorphosis larvae	
Seed, 1973	*M. edulis*	Absolute and allometric growth	N.E. England
Dare, 1976	*M. edulis*	Settlement, growth and production	Morecambe Bay, England
Incze *et al.,* 1978	*M. edulis*	Settlement, growth and survival	Maine, USA
Widdows, 1978 a,b	*M. edulis*	Body size, food, season, stress and physiology	Plymouth, England
Aguirre, 1979	*M. galloprovincialis*	Mussel biologogy	N.W. Spain
Caciun, 1980	*M. galloprovincialis*	Effect of high temperatures	
De Moreno *et al.,* 1980	*M. platensis d'Orbigny*	Lipids and fatty acids	South Atlantic waters
Hickman, 1979	*P. canaliculus*	Allometry and growth	New Zealand
Widdows *et al.,* 1979	*M. edulis*	Food and feeding	S.W. England
Bayne and Worral, 1980	*M. edulis*	Growth and production	Plymouth, England
Hickman and Illingworth, 1980	*P. canaliculus*	Condition cycle	N. New Zealand
Incze et al., 1980	*M. edulis*	Temperature, food, growth and mortality	Maine,USA
Lutz et al., 1980	*M. edulis*	Condition index and seasons	Maine,USA
Seed, 1980a	*G. demissa & B. exustus*	Shell shape and habitat	Carolina,USA
Zandee *et al.,* 1980	*M.edulis*	Biochemical composition and seasons	Dutch Wadden Sea
Almada-Villela *et al.,*1982	*M. edulis*	Effects of temperature shell growth	
Kautsky, 1982	*M. edulis*	Growth and size structure	Baltic
Mariño et al., 1982	*M. galloprovincialis*		
Loo and Rosenberg, 1983	*M. edulis*	Growth and production	W. Sweden
Ceccherelli and Rossi, 1984	*M. galloprovincialis*	Settlement, growth and production	Adriatic Sea
Dickie *et al.,* 1984	*M. edulis*	Stock, site, growth and mortality	Nova Scotia
Rodhouse *et al.,* 1984	*M. edulis*	Food, gametogenesis and growth	Killary Harbour, Ireland
Thompson, 1984	*M. edulis*	Reproductive cycle and physiological ecology	Newfoundland,Canada
Widdows *et al.,* 1984	*M. edulis*	Environmental factors and populations physiology	S. England and S. Wale
Bressan and Marin, 1985	*M. galloprovincialis*	Seasonal biochemical composition and condition index	N. Adriatic Sea
Aldrich and Crowley, 1986	*M. edulis*	Condition	Ireland

Author and year of publication	Species	Factor studied	Geographical region
Lowe and Pipe, 1987	*M. edulis*	Oil spill effect on mortality and reserve tissue	
Mallet et al., 1987a	*M. edulis*	Temperature, stock, site and growth	Nova Scotia
Page and Hubbard, 1987	*M. edulis*	Growth, temperature and food	California, USA
Widdows and Johnson, 1988	*M.edulis*	Physiological energetics: scope for growth	Norway
Bayne et al., 1989	*M. edulis*	Effect of seston concentration on feeding, digestion and growth	Plymouth, England
Mallet and Carver,1989	*M. edulis*	Stock, site, growth, mortality and secondary production	Nova scotia
Ferrán et al., 1990	*M. galloprovincialis*	Reproductive cycle	N.W. Spain
Kautsky et al., 1990	*M. edulis*	Growth and morphology on transplanted mussels	Baltic and North Sea
King et al., 1990		Proximate composition, minerals, fatty acids and sterols	
King et al., 1990	*M. edulis*	Settlement on exposed rocky shore	W. Ireland
Smaal and van Stralen, 1990	*M. edulis*	Food, growth and condition index	Scheidt, Netherlands
Ferrán, 1991	*M. galloprovincialis*	Gonadal cycle and reserve tissue	N.W. Spain
Hickman et al., 1991	*P.conaliculus*	Food and condition index	New Zealand
Mallet and Carver, 1991	*M. edulis*	Growth and mortality	Nova scotia
Navarro et al., 1991	*M. galloprovincialis*	Physiological energetics	N.W. Spain
Ferrán, 1992	*M. galloprovincialis*	Gonadal cycle and reserve tissue	N.W. Spain
Villalba, 1993	*M. galloprovincialis*	Gonad cycle and reproductive estrategies	N.W. Spain
Stirling and Okumus, 1995	*M. edulis*	Growth and production, near salmon cages	Scotland
Cáceres-Martínez and Figueras, 1998	*M. galloprovincialis*	Larval abundance	N.W. Spain
Okumuş and Stirling, 1998	*M. edulis*	Meat weight, condition index and biochemical composition	Scotland
Rajagopal et al., 1998	*Perna viridis*	Reproduction, growth rate and culture potential	Edaiyur backwaters, E. India
Sarà et al., 1998	*M. galloprovincialis*	Food and growth	S. Mediterranean Sea

Author and year of publication	Species	Factor studied	Geographical region
Akester and Martel, 2000.	*M. Trossulus*	Shell shape, dysodont tooth morphology, hinge-ligament thickness and wave exposure	
Karayücel and Karayücel, 2000	*M. edulis*	Effect of environmental factors, depth and position on growth and mortality	
Alunno-Bruscia *et al.,* 2001	*M. edulis*	Shell allometry and length-mass-density	
Budge *et al.,* 2001	*M. edulis*	Fatty acid composition phytoplankton, settling particulate matter and sediments	
Mazzola and Sarà, 2001		Effect of fish farming organic waste	Gaeta Gulf, Central Tyrrhenian, Mediterranean Sea
Freites *et al.,* 2002 a.	*M. galloprovincialis*	Effect of environemntal prameters on seeds fatty acid profiles	
Freites *et al.,* 2002 b	*M. galloprovincialis*	Lipid classes of mussel seeds	
Gökoglu, 2002		Sensory evaluation	
Orban *et al.,* 2002	*M. galloprovincialis*	Meat content, condition index and chemical composition	Italy
Babarro *et al.,* 2003	*M. galloprovincialis*	Growth patterns in biomass and size structure	N.W. Spain
Steffani and Branch, 2003	*M. galloprovincialis*	Effect on wave exposure on growth rate, condition and shell shape	
Brake *et al.,* 2004	*M. edulis*	Growth, gametogenesis and sex ratio, of triploid and diploid	
Garen *et al.,* 2004.	*M. edulis*	Growth	Pertuis Breton, France
Zotin and Ozernyuk, 2004	*M. edulis*	Growth characteristics	White Sea
Buck *et al.,* 2005.	*M. edulis*	Inshore–offshore parasite infestation on mussels	
Lauzon-Guay *et al.,* 2005	*M. edulis*	Effect of seed size and density on growth, tissue-to-shell ratio and survival	PEI, Canada

Author and year of publication	Species	Factor studied	Geographical region
Suárez *et al.*, 2005	*M. galloprovincialis*	Gonadal cycle and gametogenic stages	N.W. Spain
Waite *et al.*, 2005	*M. edulis*	Growth	PEI, Canada
Filgueira *et al.*, 2006.		Clearance rate	
Alkanani *et al.*, 2007	*M. edulis*	Fatty Acids	Notre Dame Bay, Newfoundland
Dridi *et al.*, 2007	*Crassostrea gigas*	Effect of environmental conditioin on weight, biochemical composition and gametogenic cycle	Bizert lagoon, Tunisia
Peharda *et al.*, 2007	*M. galloprovincialis*	Growth, condition index and integrated aquaculture	
Ramón *et al.*, 2007	*M. galloprovincialis*	Effect seed origin on development	Mediterranean Sea (NE Spain)
Tamayo *et al.*, 2008	*M. galloprovincialis*	Effect of food ration on mussel seed's individuals physiological rates	
Thippeswamy, 2008	*Perna viridis*	Allometry and condition index	St Mary's Island off Malpe, India
Fuentes *et al.*, 2009	*M. galloprovincialis*	Physico-chemical parameters and composition	
Ortiz-Zarragoitia and Cajaraville, 2010	*M. galloprovincialis*	Intersex and oocyte atresia	Urdaibai, S.E. Bay of Biscay
Prato *et al.*, 2010	*M. galloprovincialis*	Lipid and fatty acid compositions, feeding strategies and trophic relationships	Mar Grande of Taranto, S. Italy
Suárez Alonso *et al.*, 2010	*M. galloprovincialis*	Gonadal atresia and gametogenic cycle	Vigo, N.W. Spain
Stanković *et al.*, 2011	*M. galloprovincialis*	Trace elements, mussel quality and possible human health risk	Montenegro, Adriatic Sea
Tirado *et al.*, 2011	*M. galloprovincialis*	Culture potential	S. Spain
Martínez-Pita *et al.*, 2012	*M. galloprovincialis*	Biochemical composition, lipid classes, fatty acids and sexual hormones	S. Spain
Sánchez-Lazo and Martínez-Pita, 2012	*M. galloprovincialis*	Biochemical and energy dynamics in larval development	

Author and year of publication	Species	Factor studied	Geographical region
Gallardi *et al.*, 2014	*M. edulis*	Effects of extended ambient live holding on condition index, lipid profile, glycogen content and organoleptic properties	Newfoundland
Grienke *et al.*, 2014		Bioactive compounds and human health	
Irisarri *et al.*, 2015	*M. galloprovincialis*	Proximate composition and condition index	
Múgica *et al.*, 2015	*M. galloprovincialis*	Elevated temperature, stress biomarkers, energy metabolism and gamete development	
Oliveira *et al.*, 2015	*Mytilus sp.*	Sensory and nutrition	N.W. Spain and Algarve, Portugal

ANNEX II

List of papers

List of publication concerning the thesis

- Azpeitia, K., Ferrer, L., Revilla, M., Pagaldai, J., Mendiola, D. (2016). Growth, biochemical profile, and fatty acid composition of mussel (Mytilus galloprovincialis Lmk.) cultured in the open ocean of the Bay of Biscay (northern Spain). Aquaculture, 454, 95-108.

- Azpeitia, K., Ríos, Y., Garcia, I., Pagaldai, J., Mendiola, D. (2017). A sensory and nutritional validation of open ocean mussels (Mytilus galloprovincialis Lmk.) cultured in SE Bay of Biscay (Basque Country) compared to their commercial counterparts from Galician Rías (Spain). International Aquatic Research, 1-18.

Submitted:

- Azpeitia K, Ortiz-Zarragoitia M, Mendiola D (2017). Variability of the reproductive cycle in estuarine and coastal populations of the mussel (*Mytilus galloprovincialis* lmk.) From the SE Bay of Biscay (Basque Country).

- Azpeitia K, Rodriguez-Ezpeleta N, Mendiola M (2017). Settlement and recruitment pattern variability of the mussel (*Mytilus galloprovincialis* lmk.) From SE Bay of Biscay (Basque Country).

Azpeitia K, Urrutia MB, Mendiola D (2017). Culture production, growth rate patterns and product quality of mussel (*Mytilus galloprovincialis* Lmk.) cultured at two different depths

in the open ocean of the SE Bay of Biscay for the commercial product development.

Oral presentations from data obtained during this thesis

2014

- Aquaculture Europe 2014 (EAS) 14-17 Oct 2014. San Sebastian.
Oral presentation: "Condition and biochemical parameters of cultured mussel in shallow and deep water columns from a pilot scale commercial open ocean mussel farm in the bay of Biscay" (Katerin Azpeitia, M. Revilla, L. Ferrer, L. Ibarbarriaga and D. Mendiola).

- Aquaculture Europe 2014 (EAS) 14-17 Oct 2014. San Sebastian, Spain.
Oral presentation: "Annual Larval Dynamic of Mytilus chilensis and Environmental Factors in Ilque bay, South of Chile." (Gabriela Silva-Plaza, R. Ojeda, K. Azpeitia and R. Miranda).

- World Aquaculture Adelaide 2014. 7-11 June. (Adelaide, Australia).
"Towards a new sustainable offshore mussel farmimg industry set up in the Bay of Biscay". (Diego Mendiola, K. Azpeitia, M. Revilla, I. Galparsoro, P. Liria and M. Gonzalez.)

2013

- Aquaculture Europe 2013 (EAS) 9-12 Aug 2013. Trondheim, Norway.
Oral presentation: "Seasonal changes on reproductive cycle, growth performance and spat settlement on collector ropes near mussel Mytilus galloprovincialis beds; a case study from

the Basque Coast (South East bay of Biscay). (Katerin Azpeitia, M. Ortiz-Zarragoitia, M. Revilla and D. Mendiola).

- Aquaculture Europe 2013 (EAS) 9-12 Aug 2013. Trondheim, Norway.
"Pilot-scale commercial offshore mussel farming in the Bay of Biscay" (Diego Mendiola, k. Azpeitia, M. Revilla, I. Mentxaka, I. Galparsoro and M. Gonzalez).

- APA 2013 Positioning for profit. (WAS Asian Pacific Chapter). 10-13 Dic. Ho Chi Mihn City, Vietnam.
Oral presentation: "Seasonal changes on reproductive cycle, growth performance and spat settlement on collector ropes near mussel Mytilus galloprovincialis beds; a case study from the Basque Coast (South East bay of Biscay). (Katerin Azpeitia, M. Ortiz-Zarragoitia, M. Revilla and D. Mendiola).

2010

- Rimer 2010 "Research in Marine Environment and Resources" International postgraduate course. 3-12 Feb 2010. San Sebastian, Spain.
Oral presentation: " Longline underwater technologies for bivalve mollusk culture in open sea" (Katerin Azpeitia).

List of publication conducted independent of the thesis

The following publications resulted from data MsC Thesis (Master):

- Azpeitia, K. (2009). "Interindividual differences in physisological energetic underlying fast and slow growth of mussel (Mytilus galloprovincialis) and clam (Ruditapes phylippinarum)."Msc Thesis, University of Basque Country.

Tamayo, D., Azpeitia, K., Markaide, P., Navarro, E., Ibarrola, I. (2016). Food regime modulates physiological processes underlying size differentiation in juvenile intertidal mussels *Mytilus galloprovincialis.* Marine Biology, 163(6), 1-13.