Application of a battery of cell and tissue level biomarkers in mussels (*Mytilus sp.*) from Arctic and Subarctic latitudes: identification of confounding factors, biomarker baseline values and responsiveness for an integrated environmental health status assessment

Zelula eta ehun mailako biomarkatzaile bateriaren aplikazioa latitude Artiko eta Subartikoetako muskuiluetan (*Mytilus sp.*): faktore nahasgarrien eta biomarkatzaileen oinarri balioen eta erantzun gaitasunaren identifikazioa ingurumen osasun egoeraren ebaluazio integratua burutzeko

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1. SARRERA OROKORRA

Biojarraipen programak eta kutsaduraren ebaluazioa

Ozeano eta itsasoek Lurraren gainazalaren % 71 eta biosferaren edukiaren % 90 suposatzen dute, hori dela eta lur eta ur gezatako ekosistemek baino dibertsitate biologiko gehiago barneratzen dute (Commision of European Communities, 2005). Hala ere, mundu osoan zehar giza jarduerek sortutako kutsadurak ekosistema itsastarrak mehatxupean ditu (Adams, 2005; Garmendia et al., 2011). Kostaldeko ekosistemak isuri kroniko eta petrolio isuriak bezalako ustekabeko gertakariak direla eta bereziki afektatuak dira, organismoen osasuna, biodibertsitatea eta ondorioz ekosistemen funtzionamendua arriskuan jarriz (Islam eta Tanaka, 2004). Gaur egun kutsaduraz aparte ekosistema eta organismo itsastarrek barne eta kanpo prozesuen eragina jasaten dutela jakina da. Horien artean prozesu ekologikoak eta hauen interakzioak, arrantza guneak, aldaketa klimatikoa, habitataren aldakuntza eta eutrofizazioa egonik (Hylland et al., 2017). Honela, mundu mailan jarraipen programak bezalako estrategia desberdinak sustatu dira ekosistema itsastarretan kutsatzaileen efektuak ebaluatzeko (Melwani et al., 2014; Hylland et al., 2017; Chiu et al., 2018).

Jarraipen programa bat uretan, sedimentuetan eta/edo biotan ingurumenaren kalitate parametroen analisi sistematikoa da, area geografiko definitu batean eta denboran zehar errepikatzen dena (Goldberg et al., 1978). Itsas kutsaduraren jarraipen programen hasieran, kutsaduraren ebaluazioa uraren, sedimentuen edo biotaren konposatu kimikoen analisiaren bitartez egiten zen. Alabaina, jakina da kutsatzaileen detekzio eta kuantifikazioa metodologia analitikoen bidez ez dela nahikoa ingurugiroan duten inpaktua zehazki ebaluatzeko (Jha et al., 2000; Laane et al., 2012), kutsatzaileak ingurugiroan nahasketa konplexuetan agertzen baitira, biotan sortu dezaketen efektu toxikoen ebaluazioa zailduz (Jha et al., 2000). Honela, ekotoxikologian konplexutasun maila biologiko desberdinetako erantzun biologikoen ebaluazioa beharrezkoa da (OSPAR, 1998; Martínez-Gómez et al., 2017) eta JAMP, MED POL eta HELCOM bezalako itsas jarraipen programetan burutzen den bezala (OSPAR 1998; Viarengo et al., 2000; HELCOM, 2017). Era berean, “Itsas Estrategiaren Europar Zuzentarauak” efektu biologikoek itsas osasunaren ebaluazioan duten paper garrantzitsua (MSFD;

2008/56/EC) azpimarratzen du, ingurumen itsastarren egoera egokia zehazteko irizpideetan efektu biologikoak barneratuz (European Commission, 2008).

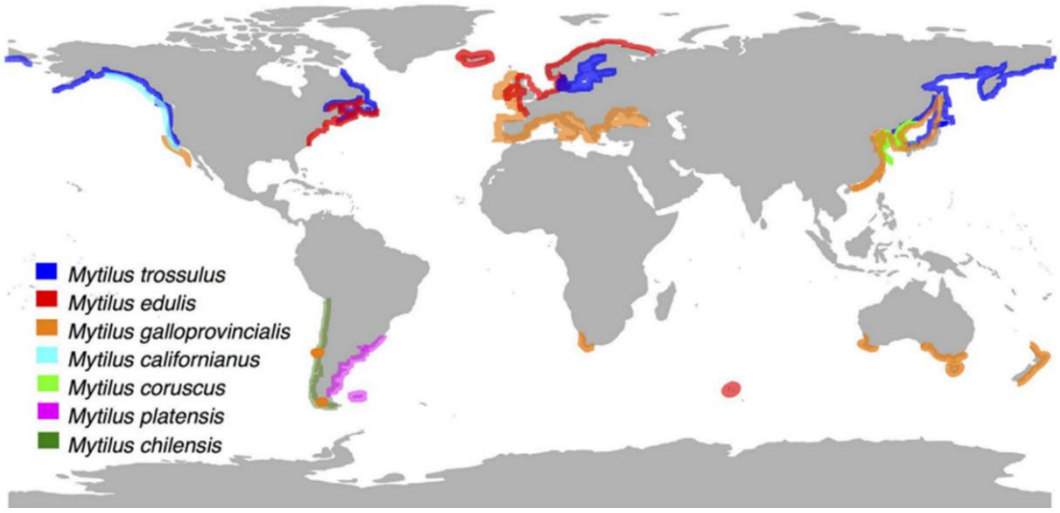
Kutsatzaileen eraginpean sortutako erantzun biologikoak ebaluatzeko biojarraipen prometan erabiltzen diren organismoak zentinela edo behaleak dute izena (Viarengo et al., 2007; Beyer et al., 2017). Organismo zentinela gisa erabili ohi diren organismoak biojarraipen prometan (Med Pol, UNEP Mediterraneoko Biomonitorizazio Programa; OSPAR Hitzarmena, UNEP/RAMOGE., eta abar) moluskuak (*Mytilus*,*Crassostrea*…), krustazeoak (*Gammarus sp.* Beste batzuen artean) eta arrainak (*Mullus .sp*., *Platichthys flesus L*., *Zoarces viviparus*, *Perca sp*., etab.) dira (Hylland et al., 2017; Viarengo et al., 2007). Organismo behaleek ezaugarri batzuk bete behar dituzte: (a) hedapen zabala, (b) kutsatzaileak biometatzeko gaitasuna, (c) egoera esperimentaletan mantendu eta ikertzeko egokitasuna, (d) kopuru handiak harrapatzeko erraztasuna, (e) mugikortasun mugatua, (f) biologia ezaguna eta (g) kutsatzaileekiko sentikortasuna izatea (Beeby, 2001; Fox, 2001; Basu et al., 2007). Organismo behaleen artean muskuiluak asko erabiltzen dira kutsatzaileen efektu biologikoak ebaluatzeko jarraipen programa itsastarretan (ICES, 2012; Beyer et al., 2017; Faggio et al., 2018) Edward Goldberg et al. (1978)-ek “Mussel Watch” izeneko programa abian ipini zutenetik. Kutsatzaileen eragin biologikoak ulertzeko, biomarkatzaileak (kutsatzaileen aurrean ematen diren erantzun biologiko goiztiarrak) itsas jarraipen prometan asko erabiltzen dira (Viarengo et al., 2007; Beyer et al., 2017), horietako asko muskuiluetan aplikagarriak izanik (UNEP/RAMOGE, 1999; ICES, 2012).

Muskuiluak organismo behale gisa

Muskuiluek organismo behale gisa duten erabilera zabala bere ezaugarri biologiko eta ekologikoak direla eta da, izan ere kutsaduraren jarraipen prometarako eta esperimentu toxikologikoetarako oso egokiak dira (Moore, 2004; ICES, 2012; Beyer et al., 2017). Muskuiluak, iragazketa bitartez elikatzen dira eta jarduera metaboliko baxua dute, beraz, hauen ehunetan aurkitu daitezkeen kutsatzaileen kontzentrazioak ingurumenean aurkitzen denaren magnitudearen isla nahiko zehatzak dira. Bestalde, nahiz eta kutsatzaileekiko sentikorrak izan, ingurumen baldintza desberdinekiko

toleranteak dira, kutsatzaile mota gehienen maila nahiko altuekiko ere. Muskuiluak zelaian biltzeko eta laborategian mantentzeko errazak dira, esperimentazioan hauen erabilera zabala ahalbidetuz. Gainera, ekonomikoki garrantzitsuak diren organismo sesilak dira, geografikoki oso hedatuta egonik. *Mytilus* generoko itsasertzeko komunitate arrokatsuetako osagai garrantzitsuenetarikoak dira ipar eta hego hemisferioko ur hotz eta epeletan (1. irudia). Hiru taxon aurkitzen dira (*M. galloprovincialis*, *M. edulis* eta *M. trossulus*) Europan (Hilbish et al., 2000; McDonald et al., 1991). Hiru taldeek identitate genetiko nahiko desberdintzatuak mantentzen dituzte mundu mailan, neurri handi batean taxonak probintzia biogeografiko ezberdinetara egokituta daudelako (Gardner, 1996). Hala ere, probintzia biogeografiko batetik besterako trantsizio eremuetan, hibridoak aurkitu daitezke (Brooks et al., 2015; Gardner eta Thompson, 2001).

Muskuiluak (*Mytilus spp.*) marearteko guneetatik marea azpiko guneetara hedatzen dira, estuarioetatik baldintza itsastarretararte, eta kostaldeko gune babestuetan eta olatuen efektua jasaten duten guneetan aurkitu daitezke (Gosling, 2004). Marearteko eremuan, muskuiluen goi banaketa muga faktore fisikoek gobernatzen dute. Harrapariek berriz, beheko muga ezartzearen arduradunak dira nagusiki (Seed eta Suchanek, 1992).



1. Irudia: *Mytilus* generoko muskuilu itsastarren distribuzioa (Gaitán-Espitia et al., 2016)

Muskuiluen ehun bigunak analisi kimiko zein haragi baldintza indizeetarako erabiltzen da ingurumenaren osasunaren ebaluazioan. Gainera, organo desberdinak,

hala nola oina, mantua, zakatzak eta liseri-guruina, bereizita erabiltzen dira biomarkatzaile desberdinak aplikatzeko (ICES, 2012). Oinak lokomozio gaitasuna ematen die muskuiluei, batez ere fase jubenilean. Oina proportzionalki oso tamaina handia dauka, galtzerdi itxurakoa da, eta hemolinfa espazio zabal bat inguratzen duten gihar geruza zirkular eta longitudinalek osatzen dute.

Bibalbioetan, mantua oskolaren barrukaldetik animalia osoa inguratzen duten bi lobuluz osatuta dago, hemolinfa hodiak, nerbioak eta ertzeetan bereziki garatutako muskuluak dituen ehun konektiboz osatuta dago. Mantuaren ertzak pigmentatuta egon ohi dira, eguzki-erradiazioaren eragin kaltegarrietatik babesa eskaintzen duenak (Seed, 1971). Gametoak mantuan ugaritzen dira eta ziliodun kanaletatik eramaten dira gonoduktu parekatuetara eta mantuaren barrunbean isurtzen dira. Gametoak askatu ondoren mantua mehe eta gardena da. Itsas ekosistemen osasunaren ebaluazioan gametoen garapena aztertzen da muskuiluen osasunari eta fisiologiari buruzko informazio osagarria ematen duelako (ICES, 2011). Gainera, normalean 1:1 ar eme erlazioa duten arren sexu ratio hauen aldaketak disruptore endokrinoen kutsadura ekosisteman iradoki lezake (Ortiz-Zarragoitia et al., 2011).

Bibalbioen ugalketa-zikloa ebaluatzeko metodo fidagarrienak mantuaren gertakin histologikoetan oinarritutakoak dira (Hines et al., 2007). Halako gertakinetan gonaden garapen-etapa desberdinak identifikatu daitezke, Ortiz-Zarragoitia et al. (2011) deskribatu bezala (4. irudia):

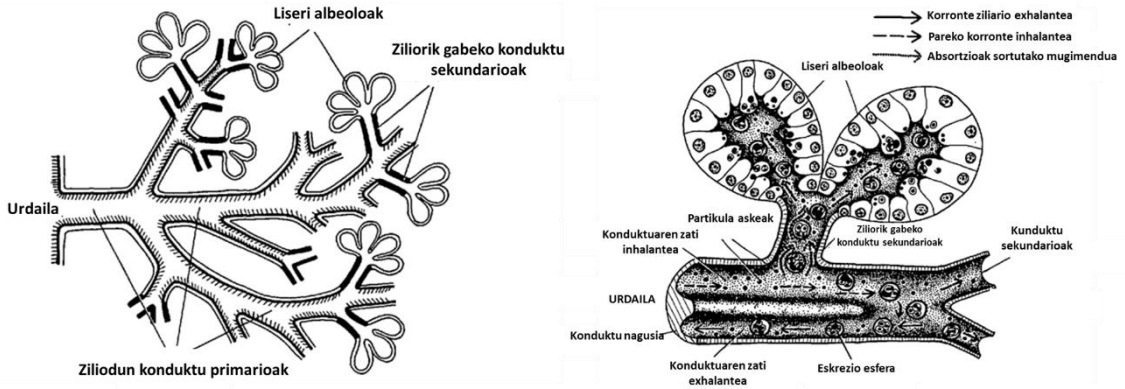
- I. etapa. Atsedenaldia. Gonada inaktiboa edo ez diferentziatua.
- II. etapa. Gametogenesi goiztiarra. Gametogenesia hasi da, baina ez dago gameto heldurik.
- III. etapa. Gametogenesi aurreratua. Gameto helduak eta heldugabeak antzeko proportzioetan aurki daitezke.
- IV. etapa. Gonada heldua. Folikuluak obuluz edo espermaz beterik.
- V. etapa. Errutea. Folikuluak partzialki hutsik agertzen dira.
- VI. etapa. Errute-ostea. Folikuluak hutsik edo gameto hondarrekin agertzen dira.

Ugalketa zikloak ingurumen faktoreen aurrean malgua da (tenperatura, elikagaien eskuragarritasuna), geografikoki erlatiboki hurbil dauden muskuilu

populazioen arteko desberdintasunak sortuz edo/eta urteko bariazioak eraginez populazio berdinaren barnean. Hala ere, kutsatzaileek aldaketa nabarmenak eragin ditzakete ugalketa zikloan (Ortiz-Zarragoitia et al., 2011). Orokorrean, *Mytilus sp.* muskuiluen gonaden garapena neguaren eta udaberriaren bueltan hasten da uraren tenperatura eta elikagaien eskuragarritasuna handitzen direnean, errutea udaberria aurreratuagoa dagoenean gertatu ohi da (Gosling, 2004; Beyer et al., 2017). Elikagaien eskuragarritasunaren arabera, gonadak berreskuratzeko eta umatzeko sekuentzia berriak uda amaieran gertatu daitezke. Bestela gonaden birxurgapena ematen da erreserba ehunen garapenarekin batera. Ziklo gametogeniko berria uda amaieran hasten da (Garmendia et al., 2010; Ortiz-Zarragoitia et al., 2011). Mantua mantenugaien erreserbak metatzeko organo nagusia da, glukogenoa batez ere (Gosling, 2004). Erreserbak udan zehar metatzen dira eta udazkenean eta neguan erabiltzen dira gametoak eratzeko. Muskuiluak planktonez eta beste itsas izaki mikroskopikoez elikatzen dira. Zakatzak, “ctenidia” ere deitzen direnak, lauak dira, homorhabdikoak (grekerazko *homos*, bera; *rabdos*, haga; serie uniformeak), ez-plikatuak eta filibrankoak. Zakatzak gasen elkartrukea eta elikadura gauzatzen dute. Hauen gainazal zabal eta meheak eta hemolinfa hornidura aberatsak gas- trukerako egokiak izatea dakar. Hemolinfa desoxigenatua giltzurrunetatik zakatzetara eramaten da zakatz-hodi aferentearen bidez. Filamentu bakoitzak hodi honen adar txiki bat jasotzen du. Filamentuak, funtsean, hodi hutsak dira eta barruan hemolinfak zirkulatzen du. Gas-trukea filamentuen horma meheetan zehar gertatzen da. Filamentu bakoitzeko hemolinfa oxigenatua zakatz hodi eferenteren bidez giltzurrunera eta bihotzera eramaten da. Zakatzetan oxidazio prozesuak ugariak dira, disolbatutako kutsatzaileen sarrera gune nagusia zakatzak bait dira. Ondorioz urak garraiatutako kutsatzaileen eraginpean dagoen lehen organoa da (Vidal-Liñán eta Bellas, 2013).

Muskuiluetan, elikadura zakatzetan hasten da. Zakatzak, mantuaren barrunbean zehar ponpatutako uretan suspenditutako partikulak traktu ziliar ezberdinekin hartzen dituzte, garraio mekanismo hidromekaniko eta mukoziliarra erabiliz (Gosling, 2004). Zakatzek mantuaren barrunbea ganbara inhalante eta exhalanteetan banatzen ditu. Irekiera inhalantetik sartzen den ura ganbara exhalantera eramaten da zakatz eta mantuaren gainazaleko zilioen bidez, eta irekidura exhalantetik irteten da. Bi

irekiguneek velum (mantuaren barneko tolesdura) gihartsuak dituzte, mantuaren barrunbean zehar ur-fluxua erregulatzen dutenak. Esekiduran dauden partikulak ezpainetako palpoen oinarrian dagoen zirrikitu batetik sartzen dira ahoan. Ahoak, eta urdailera doan hestegorriak, gaineztadura-epitelio ziliatua dute, mukopolisakarido azidoak eta neutroak jariatzen dituzten mukozitoen bitartez, animalia elikatzen ez denean ere (Beninger eta Le Pennec, 1991). Hestegorriak ez du liseri-funtziorik, materiala zilioen bitartez liseri-guruinean dagoen urdailera heltzeko soilik balio du. Izenak adierazten duen bezala, liseri-guruinaren funtzio nagusia liseriketa da. Urdaila zaku lau bat da, eta bertara hestegorria irekitzen den aurreko muturrean eta hestea irteten da. Estilo kristalinoa urdailaren atzeko muturretik proiektatzen da urdailaren zoruan zehar eta ezkutu gastrikoaren aurka eusten da. Irensten diren partikulak estilo kristalinotik askatutako liseri-entzimekin nahasten dira (Gosling, 2004). Nahasketa eta liseriketa zelulaz kanpoko prozesuetan liseritzear dagoen materiala urdaileko eremu handiak estaltzen dituzten traktu ziliatuen eraginpean daude. Bibalbioen liseri-guruinean, urdaila amaiera itsuko albeoloetan adarkatzen da konduktu ziliatu sekundarioen ondoren (2. Irudia). Hodi horien barruan bi norabidetako fluxu jarraitua dago (2. Irudia): materiala hodietan edo albeoloetan sartzen da zelula barneko liseriketa eta xurgapena gauzatzeko eta hondakinak urdaila eta hesteetara kanporatzen dira.

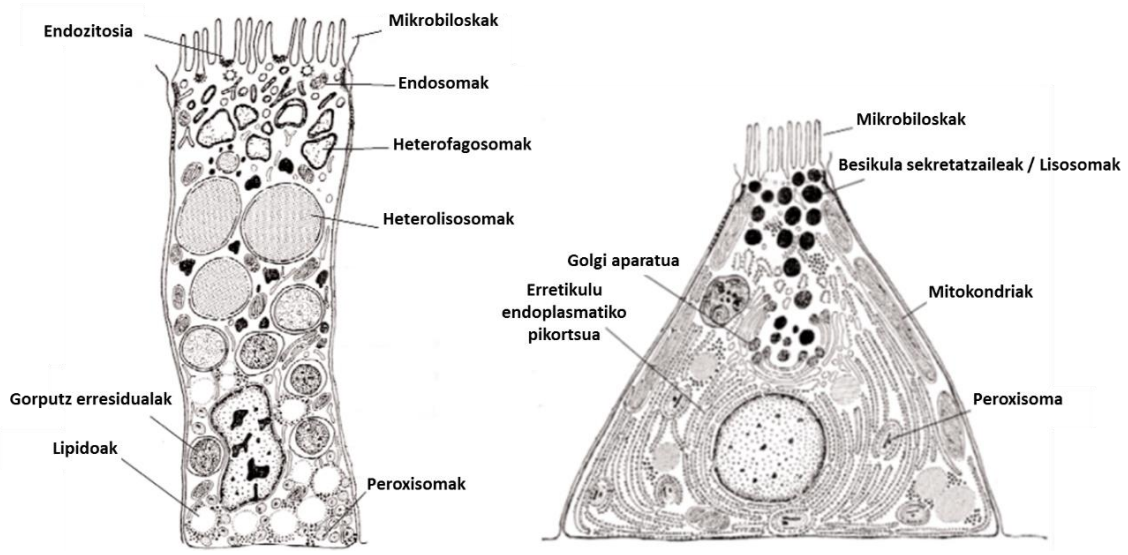


2. Irudia: Ezkerrean, bibalbioen liseri-guruinaren konduktu sistema. Eskubian, bibalbioen liseri-guruinaren ebaketa non absortzioa eta zelula barneko liseriketa ikusten diren. Owen (1955)-tik moldatua.

Liseri-albeoloen epitelioa bi zelula motaz osatuta dago, liseri-zelulak eta zelula basofiloak (Morton, 1983) (3. irudia). Baldintza normaletan, liseri zelulak ugarienak dira (Marigomez et al., 1990; Zaldibar et al., 2008). Zutabe formakoak dira, ondo garaturiko

sistema endolisosomikoa dute eta elikagaien liseriketa intrazelularra gauzatzen (Morton, 1983; Owen, 1972). Liseriketa entzima hidrolitikoak dituzten lisosomen barruan gertatzen da. Lisosomak hidrolasa azidoak (pH 4,5-5,5) dituzten ia eukarioto guztietan oso kontserbatuak dauden funtzio anitzeko organuluak dira eta elikagaien zelula barneko liseriketa burutzen dute (Allison, 1969). Konpartimentu endolisosomikoa etengabe fusionatzen den besikula sistema bat da (a) Golgi aparatutik ateratako besikula jariatzaileen entzima hidrolitiko berriak jasotzeko eta; (b) autofagosomekin eta endosoma berantiarrekin batzeko (Owen, 1973). Horrela, organulu kaltetuen (mitokondrioak eta erretikulu endoplasmatikoa) degradazioan zelulen ziklo autofagikoaren fase gisa (Klionsky eta Emr, 2000; Moore et al., 2006) eta endozitosiaren bidez irensten diren materialen liseriketan parte hartzen dute (Moore, 1985).

Zelula basofiloak piramide formakoak dira (3. Irudia) eta hauen zitoplasmaren zati handi bat erretikulu endoplasmatiko pikortsuaz (ER) eta Golgi gorputzez dago osatuta, eta horrek adierazten du zelula hauek proteinen sintesian dutela funtzio nagusia. Albeoloen lumenera zelulaz kanpoko liseriketarako entzimen jariapenean garrantzia izan dezakete, baina jariatze zelulen eginkizun zehatza ez dago argi (Weinstein, 1995). Lekube et al. (2000) eta Izagirre eta Marigómez (2009) entzimen transferentzia lisosomikoa gerta litekeela iradoki zuten zelula basofiloetatik liseri-zeluletara. Horrela, zelula basofiloek, ER ondo garatua dutenek, hidrolasa azidoak transferituko lituzkete liseri-zeluletara, ER eta Golgi gutxiago garatuta dituztenak eta entzima lisosomikoen eskari altua dutenak asko garatutako sistema endo-lisosomikoa hornitzeko, neurri handi batean liseriketa/marea ziklo guztietan berritu behar dena. Liseriketa prozesuaz gain, liseri-guruinak xenobiotikoen detoxifikazioan parte hartzen du eta kutsatzaileak metatzen ditu. Hori dela eta, liseri-guruina itu-ehun nagusienetarikoa da toxikologian biomarkatzaileen ikuspegia aplikatzen denean (ICES, 2012). Horrela, biomarkatzaileak hobeto interpretatu ahal izateko liseriketa prozesua eta liseri-guruinen funtzionamendua ulertzea garrantzitsua da.



3.Irudia: Bibalbioen liseri-epitelioa osatzen duten bi zelula moten ultraestrukturaren ikuspegia. Ezkerrean, liseri-zelulak. Eskubian zelula basofilikoak. Owen (1973)-tik moldatua.

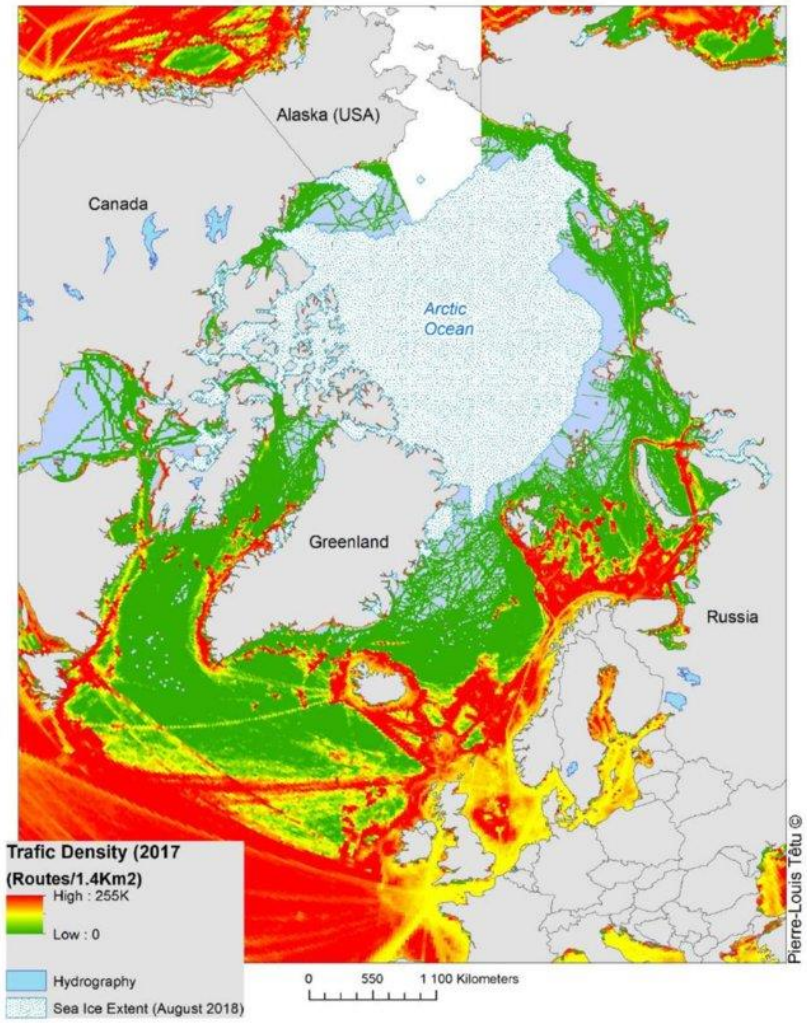
Ipar Ozeano Atlantikoa, Ozeano Artikoa eta Itsaso Baltikoa: muturreko baldintzak eta petrolioaren mehatsua

Azken urteotan, petrolio ustiatze-gune berriak eraiki dira Itsaso Baltikoan eta Ipar Ozeano Atlantikoan, itsasontzi bidezko petrolio garraioa areagotuz (4. Irudia), eta ondorioz, istripuak gertatzeko arriskua emendatuz. 1977. urtetik abiatuta, itsaso hauetan, hainbat istripu gertatu dira petrolio isuri garrantzitsuak sortuz (Marine oil Information Gateway, 2005). Hau horrela izanik, ikerketa gunean itsas ekosistemetarako petrolio isuriak mehatxu garrantzitsuenetarikoen artean daude (Kostianoy et al., 2004). Petrolio isurien inpaktua dela eta (iraganean, gaur egun edo etorkizunean) argi dago Ipar Atlantikoa eta itsaso Baltikoa bezalako interesezko guneetan beharrezkoa dela jarraipen programak gauzatzea.

Erregai fosilen eskaera gero eta handiagoa izanik, petrolio erauzketak eta ozeanoetako garraioa nabarmen hazi dira azken hamarkadatan. Ondorioz, petrolio isurketak gertatzeko arriskuak areagotzen ari dira. 2010 eta 2018 artean, 59 petrolio isuri handi gertatu ziren mundu osoan, eta, ondorioz, 163.000 tona petrolio inguru isuri

ziren itsas ingurunera (ITOPF 2019). Gaur egun, itsaso artiko eta subartikoetan munduan aurkitu gabeko petrolioaren % 13-ra arte dagoela kalkulatu da (Camus eta Smit, 2019).

Ingurune hotzetan petrolio-isurien adibiderik ezagunena Alaskan 1989-an gertatu zen Exxon Valdez-eko petrolio-isuria izan zen. North Slope petrolio astunaren 41,6 milioi litro (37.000 tona) isuri ziren uharteetako itsasertzetik gertu (Atlas eta Hazen, 2011). Isuriaren ondoren gertatutako ekaitzek petrolioaren barreiapena asko emendatu zuten itsasertzaren % 15 estalduz *Prince William Sound* eta Alaskako Golkoan zehar (Short et al., 2004). Gertaera honek eragin handia izan zuen inguruko arrain eta ornogabe espezietan. Esaterako, Ozeano Bareko sardinzar (*Clupea pallasi*) larben artean anomalia tasa handiagoak behatu ziren olioztaturiko eremuetan eraginik gabeko eremuekin alderatuta (Faksness et al., 2020).



4. Irudia: Ipar Itsasoko eta Itsaso Baltikoko itsasontzi trafikoa (Lasserre eta Têtu, 2020).

Itsas ekosistema artiko eta subartikoen berezitasunak

Ingurune artiko eta subartikoak 50° iparraldeko paralelotik haratago dauden eskualdeak dira. Ingurune hauek gauerdiko eguzki eta egun osoko iluntasun aldiak izaten dituzte eta hilabete beroenek ez dituzte batez beste 10°C gainditzen (Hoberg et al., 2012). Ekosistema Artiko eta subartikoak aurkezten dituzten muturreko baldintzek direla eta bereziak dira izaki bizidunentzat. Neguan, ozeanoaren gainazala izoztu egiten da. Izotz estalki hau oso azkar urtzen den geruza mehetik hasi eta 3 m-ko lodiera izan dezakeen eta urte askotan iraun dezakeen izotz geruza lodia izatera ailegatu daiteke. Itsasoaren gainazala urte osoan zehar izotz-geruzaz estalita egon daitekeen arren, ingurune artiko eta subartikoak bizitzaz gainezka daude. Ekosistema hauek bi mila alga-espezie, hamarnaka mila mikrobio eta gutxi gorabehera bost mila animali-espeziek osatzen dituzte (Meltofte et al., 2013). Beste edozein ekosistema bezala, ekosistema hauek elika-sare konplexuetan antolatuta daude, mikrobio, fitoplankton, zooplankton, makroornogabe, arrain, itsas hegazti eta ugaztunen arteko elkarreraginekin.

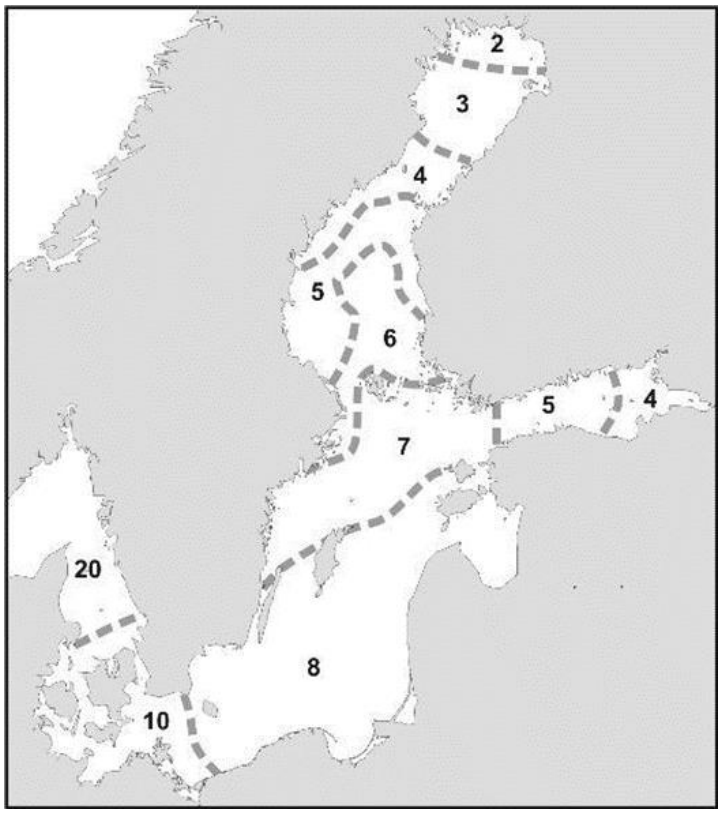
Itsas ekosistema artikoak oso sentikorrak dira giza jarduerek eragindako aldaketekiko. Ustiapen handiko arrantza komertzialaren presio larriak, isurketek eragindako kutsadurak, atmosferan ematen den distantzia luzeko kutsatzaileen transferentziak, kutsadura akustikoak, petrolioa eta mineralen ustiapenak, tenperatura aldaketak, ozonoaren murrizketak, ontzien trafikoa areagotzeak eta beste faktore batzuek zuzenean edo zeharka eragiten dute ekosistema artikoen orekan (Clarke eta Harris, 2003; Jenssen, 2003, 2006; Huntington, 2009)

Ozeano Artikoko itsas ingurunea oso sentikorra da kutsadura kimikoarekiko, hala nola petrolio-isuriak eta hiri- eta industria-hondakinen isurketak, izotz-estalkiak garbiketa zaildu edo ezinezko bilakatzen baitu. Gainera, fauna sentikorragoa izan daiteke klima hotzetan isuritako hainbat kutsatziale (olioa bereziki) poliki degradatzen baitira. Itsasoan isuritako petrolioa jarduera desberdinen bitartez (irenstea, arnasketa, xurgatzea) animalietan sartzen da eta mehatxu zuzena eta hilgarria da bizidunentzat (Dietz et al., 2019).

Itsaso Baltikoaren berezitasunak

Itsaso Baltikoa Europako iparraldean dago, 10°-30°E eta 54°-66°I artean. Itsaso Baltikoa orokorrean sakonera txikikoa dela kontsideratu daiteke, izan ere, batez besteko 55 m-ko sakonera baitu. Arrorik sakonenek gehienez 495 m-ko sakonera dute eta Baltic Proper izeneko eskualdean daude. Itsaso Baltikoak itsasoak berak baino lau aldiz handiagoa den eremuko ibaietako ur geza jasotzen du. Ipar Atlantikoarekin Danimarkako itsasarte estu eta azalekoarengatik soilik dagoenez konektatuta, Itsaso Baltikoko ur-trukea mugatuta da. Inguruko hidrografiaren ezaugarri berezien artean, hegoaldetik iparraldera uraren gazitasunaren beherakada (5. Irudia), eta 60 eta 100 m bitarteko sakoneran nagusi den haloklina iraunkorra daude. Haloklinak, zeinak gainazaleko ura ur gezagoa sakoneko geruza gaziagotik bereizten duen, ur sakonen oxigenazioa Itsaso Atlantiko ur gazien noizbehinkako sarrerara mugatzen du (Leppäranta eta Myrberg, 2009). Baldintza hipoxikoak edo anoxikoak haloklinaren azpiko eremuetan egon ohi dira, hondoko komunitateen bizitza mugatuz (Ojaveer et al., 2010). Udan ur-zutabea ere tenperaturaren arabera estratifikatzen da, eta 10-30 m-ko sakoneran dagoen termoklinak goiko ur-geruza epela azpiko ur hotzetatik bereizten ditu. Udazkenean azaleko uraren hozte azkarrak geruzak nahastea ahalbidetzen du. Neguan izotz-estaldura partziala edo osoa sortzen da itsasoaren iparraldean, Finlandiako golkoan eta Botniako itsasoan 2-4 hilabete irauten du. Urtean zehar ematen diren tenperatura gorabehera nabarmenak bereizgarriak dira batez ere iparraldean. Gainazaleko ura berotzearekin, udaberri hasieran ikusitako zerotik hurbileko tenperaturetatik, gutxi gorabehera 20°C tenperaturetara iristen da uztailean (Leppäranta eta Myrberg, 2009). Gainazaleko uren bat-bateko hozteak *upwelling* edo azaleratze prozesu batek eragiten du, ur-masak aldi baterako nahasten dituena norabide bertikalean. *Upwelling* prozesuak normalean haizeek eragiten dute eta gehienetan kostaldetik hurbil dauden uretan gertatzen da (Omstedt et al., 2014). Itsaso Baltikoko fauna itsas eta ur gezako espezieak osatzen dute, eta sarritan organismoen banaketa-eremua zehazteko faktore kritikoena gazitasuna da. Gainera, goi mailako organismoen biodibertsitatea nabarmen txikiagoa da benetako itsas edo ur gezako ekosistemekin alderatuta, eta ezinbesteko ekosistemetako zerbitzu asko funtsezko

espezie bakarrak gauzatzen ditu (Koivisto eta Westerbom, 2010; Ojaveer et al., 2010). Neguan ekosistema osoa moteltzen duten argi mugak eta tenperatura baxuko baldintzek ere organismoen biziraupena zalantzan jartzen dute. Udaberriko ekoizpen primarioaren maximo estentsiboak eta argi-ordu ugariak eta udan uraren tenperatura igotzeak organismo heterotrofoen elikadura eta metabolismoa aktibatzen dituzte. Aipatutako ezaugarriek Baltikoa asalduren aurrean oso sentikorra bihurtzen dute, eta itsasoak eutrofizazioa, alga toxikoen loratzeak, substantzia arriskutsuak, azidifikazioa, hondo anoxikoen hedatzea eta espezie arrotz kaltegarrien sarrerak jasaten ditu (HELCOM 2010 a,b). Itsaso Baltikoko osasuna sailkapen integratzaileko hainbat tresna erabiliz ebaluatu da (HELCOM 2010a). Substantzia arriskutsuen kontzentrazioei, biodibertsitateari eta eutrofizazioari dagokionez, azkenaldian Finlandiako Golkoaren eta “Baltic Proper”-en egoera "moderatua", "eskasa" edo "txarra" gisa sailkatu da, bost urratseko irizpideetan, "altua"-tik "txarra"-era. Ebaluatutako kategoriei dagokienez, egoera "ona" Botniako Golkorako bakarrik erregistratu zen (HELCOM 2010b). Ekosistemen osasunaren ebaluazio eta sailkapen integratuak Finlandiako kostaldeko eremuaren eta “Baltic Proper”-en eremu gehienen egoera "eskasa"-tik "txarra"-ra definitu zuten (HELCOM 2010b). Baltikoko Itsasoko Ekintza Planaren (BSAP) akordioaren helburuak substantzia arriskutsuei dagokienez honako hauek dira: 1) "Oinarri mailetatik gertu dauden kontzentrazioak", 2) "Animalien osasun-arazorik ez" eta 3) "Arrain guztiak jateko seguruak" (HELCOM 2007). Helburu horiek lortzeko ebaluazio integratuak lan asko geratzen dela erakutsi zuen. Substantzia arriskutsuen kontzentrazioen jarraipena indartu behar da eta HELCOM-eko efektu biologikoen monitorizazioaren garapena adostu behar da HELCOM-eko Alderdi kontratatzaile guztietan (HELCOM 2010b).

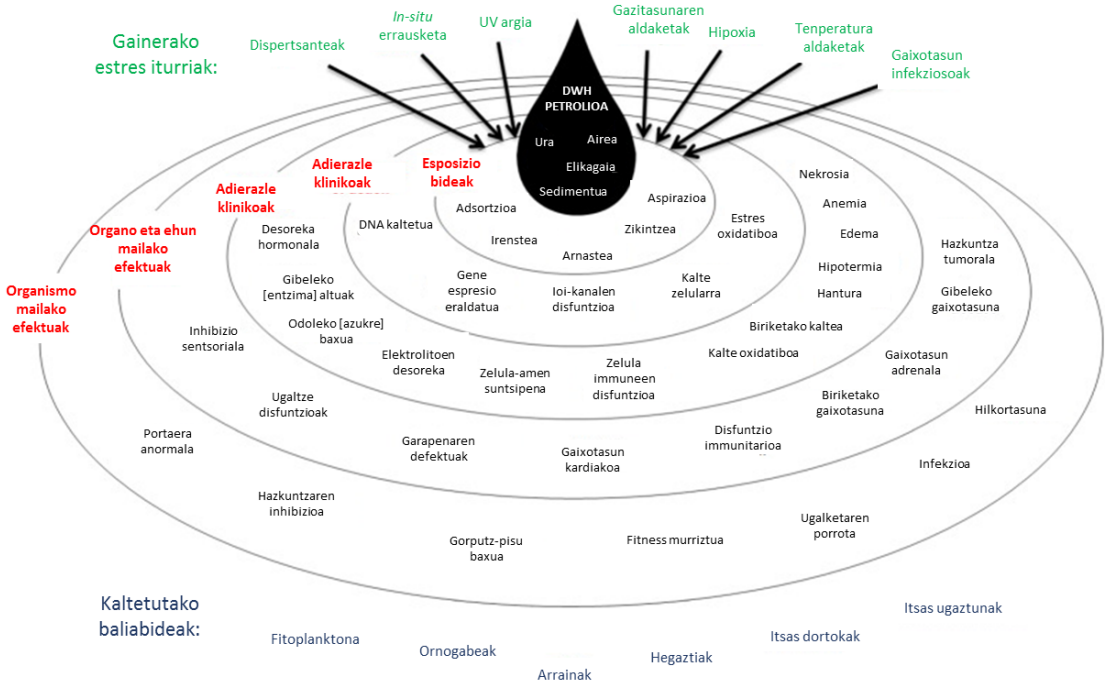


5. Irudia: Itsaso Baltikoko gazitasun gradientea (PSU). S. Welsawski, (http://www.marinespecies.org/-tik berreskuratua: 2021/12/09)

Biomarkatzaileak: osasun egoeraren adierazleak

Arestian azaldu bezala, muskuiluak kutsatzaileen aurrean erantzun biologikoak ebaluatzeko zelai zein laborategiko ikerketetan sarritan erabiltzen diren organismoak dira (Beyer et al., 2017; Faggio et al., 2018). Biomarkatzaile kontzeptua hasieran diagnostiko medikoan aplikatu zen gizakien egoera edo gaixotasun jakin baten adierazle gisa (Paone et al., 1980), eta 1990eko hamarkadaren hasieran, oso erakargarria bihurtu zen ingurumen-ikerketetan (Depledge eta Fossi, 1994; Peakall, 1994). Ingurumen-toxikologian, biomarkatzaileak maila biokimiko, zelular edo ehun mailako neurketatzat onartzen dira, zeinak kutsatzaileen presentzia (esposizio-biomarkatzaileak) eta/edo erantzunaren magnitudea adierazten duten (efektu-biomarkatzaileak) (McCarthy eta Shugart, 1990). Abisu goiztiarreko erantzun hauek konplexutasun biologikoko maila baxuetan gertatzen dira eta iragarpenak egiteko aukera ematen dute, epe luzeagoan,

antolaketa biologiko-maila konplexuagoetan (6. Irudia) gertatuko diren iragarpenak egiteko aukera ematen dute. Oso gomendagarria da maila biologiko desberdinetako biomarkatzaile bateria bat erabiltzea, esate baterako, biokimikoa, zelularra eta ehun mailakoa, izan ere, biomarkatzaile indibidual batek kutsatzaileen presentziari buruzko informazio partziala baino ez du ematen (UNEP/RAMOGE, 1999).



6. Irudia: "Deep Water Horizon" petrolio isuriaren efektuak ingurugiroan, Beyer et al. (2016)-etik moldatua.

Ipar Ozeano Atlantikoan, Ozeano Artikoan eta Itsaso Baltikoan erabilitako biomarkatzaileak zelai ikerketetan: hutsuneak bilatzen

Azkeneko bi hamarkadatan Itsaso Baltikoan eta Ipar Ozeano Atlantikoan muskuiluen biomarkatzaileak erabiliz egindako zelai ikerketek (1. Taula) hainbat erantzun biologikoen bateriak erabili dituzte. Itsaso Baltikoan adibidez, gehienbat biomarkatzaile biokimikoak eta zelularrak aplikatu dira. Maila txikiagoan, biomarkatzaile molekularrak eta fisiologikoak erabili dira, eta gutxien erabili izan direnak ehun-mailako biomarkatzaileak eta histopatologia dira. Ipar Ozeano Atlantikoan, berriz, gehien erabili diren biomarkatzaileak maila zelularrekoak dira, eta hauen atzetikbiomarkatzaile fisiologiko eta biokimikoak. Analisi histopatologikoak Itsaso Baltikoan baino hedatuagoak daude, baina ehun mailako biomarkatzaileak ez dira maiz erabiltzen.

1. Taula: Ikerketa eremuetan azkeneko bi hamarkadetan egindako biomarkatzaileetan oinarritutako ikerketak.

ITSASOA	ARGITALPENA	EREMU GEOGRAFIKOA	BIOMARKATZAILEAK
Itsaso Baltikoa	Leiniö eta Lehtonen (2005)	Finlandiako kostaldea	MT, AChE, CAT, GST
	Baršiene et al. (2006)	Lituaniako kostaldea	MN, NL, LMS, AChE
	Lehtonen et al. (2006)	Finlandiako kostaldea	AChE, GST, CAT, MT
	Schiedek et al. (2006)	Alemaniako kostaldea	LMS, AChE, MN, MT, EROD, DNA aduktuak
	Dabrowska et al. (2013)	Gdańsk-eko golkoa	AChE, GST, CAT, GR, SOD, LPO, CI
	Turja et al. (2014)	Suediako kostaldea	LMS (NRRT), SOD, CAT, GR, LPO, GST, AChE, MN, Thiamina edukia, Erantzun bioenergetikoak, Jarduera fagozitikoa, Erritmo kardiakoa
	Höher et al. (2015)	Botniako golkoa	Jarduera fagozitikoa, Haemozito kopurua, Kaspasa jarduera, Jarduera hemolitkoa
	Höher et al. (2015)	Danimarkako kostaldea	CI, Jarduera fagozitikoa, Kaspasa jarduera Jarduera hemolitkoa
	Turja et al. (2015)	Finlandiako golkoa	SOD, CAT, GR, LPO, GST, AChE, Genotox, Bioenergetika, LMS (NRRT)
	Lehtonen et al. (2016)	Artxipelago itsasoa (Finlandia)	AChE, MT, GST, CAT, GR, SOD
	Smolarz et al. (2017)	Gdańsk-eko kostaldea	HP, Gonadaren atresia
	Larsson et al. (2018)	Suedia, Polonia eta Finlandiako kostaldeak	Lipido edukia, Atrofia indizea, HP, MN, BMI, GSI
	Lastumäki et al. (2020)	Bornholm arroa	CI, CAT, GR, GST, LMS (NRRT), Glikogeno edukia, LPF, NL, Genotox, Citotox, CEA
Ipar Ozeano Atlantikoa	Einsporn et al. (2005)	Wadden-eko itsasoa (Alemania)	LMS, NADPH
	Halldórsson et al. (2005)	Islandia hegomendebaldea	SFG
	Dondero et al. (2006)	Norvegia hegoaldea	LMS, LPF, NL, LSC, Ca^{2+}-ATPase, CAT, MT
	Zorita et al. (2006)	Norvegia hegoaldea	LMS, LPF, NL, LSC, Vv_{BAS}, HP
	Da Ros et al. (2007)	Islandia hegomendebaldea	NL, LSC, LPF, LMS, MT, HP
	Aarab et al. (2008)	Norvegia hegoaldea	LPF, LMS, HP
	Halldórsson et al. (2008)	Islandia hegomendebaldea	DNA harien hausturak, Erritmo kardiakoa, Elikatze ratioa
	Hylland et al. (2008)	Iparraldeko itsasoa	LMS (NRRT eta HC), MN, HP, Vv_{BAS}, MLR/MET
	Sundt et al. (2011)	Norvegia hegoaldea Iparraldeko itsasoa)	MN, LMS (NRRT)
	Beyer et al. (2013)	Norvegia Iparraldea (Melkøya)	(Zelai-ikerketan) LMS (NRRT), Haemozitoen nukleoen osotasuna, MN
	Nahrgang et al. (2013)	Norvegia Iparraldea (Tromsø)	CAT, GPX, GST, LPO, TOSC, EROD, LMS (NRRT)
	Brenner et al. (2014)	Iparraldeko itsasoa (Alemania)	CI, Parasitoen edukia, LMS, Glikogeno edukia, LPF, NL
	Helmholz et al. (2015)	Iparraldeko itsasoa (Alemania)	Proteomika
	Helmholz et al. (2016)	Iparraldeko itsasoa (Alemania)	CI, GSI, Energia ekonomiari lotutako parametroak, MET jarduera
	Storhaug et al. (2019)	Norvegia iparraldea (Tromsø)	CI, LMS (NRRT), LPO, CAT, GST, TOSC-OH, TOSC-ROO

Kolore kodea: Berdez, biomarkatzaile molekularrak; Laranjaz, biomarkatzaile biokimikoak; Urdinez, biomarkatzaile zelularrak; Beltzez, biomarkatzaile fisiologikoak; Gorriz, ehun mailako biomarkatzaileak eta histopatologia. Laburdurak: MN: Mikronukleoen presentzia, NL: lipido neutroen kontzentrazioa, LMS: Mintz lisosomikoaren egonkortasun testa, NRRT: Gorri neutroaren erretentzio denbora, AChE: Azetilkolinesterasa, MT: Metalotioneina edukiak, CAT: Katalasa jarduera, GST: Glutation-S-Transferasaren jarduera, EROD: Ethoxyresorufin-O-deethylasa jarduera, GR: Glutation erreduktasa jarduera, SOD: Superoxido dismutasa jarduera, LPO: Lipido peroxidazio maila, CI: Egoera indizea, HP: Histopatologia, BMI: Gorputz-masaren indizea, GSI: Indize gonadosomatikoa, LPF: Lipofuszinak, CEA: Zelularen energiaren distribuzioa, SPF: Hazkuntzarako irismena, LSC: Lisosomen egitura aldaketak, MET: Elektroi garraio sistema mitokondriala.

Ikerketa honetan erabilitako gako biomarktazaileak

Biomarkatzaile lisosomikoak

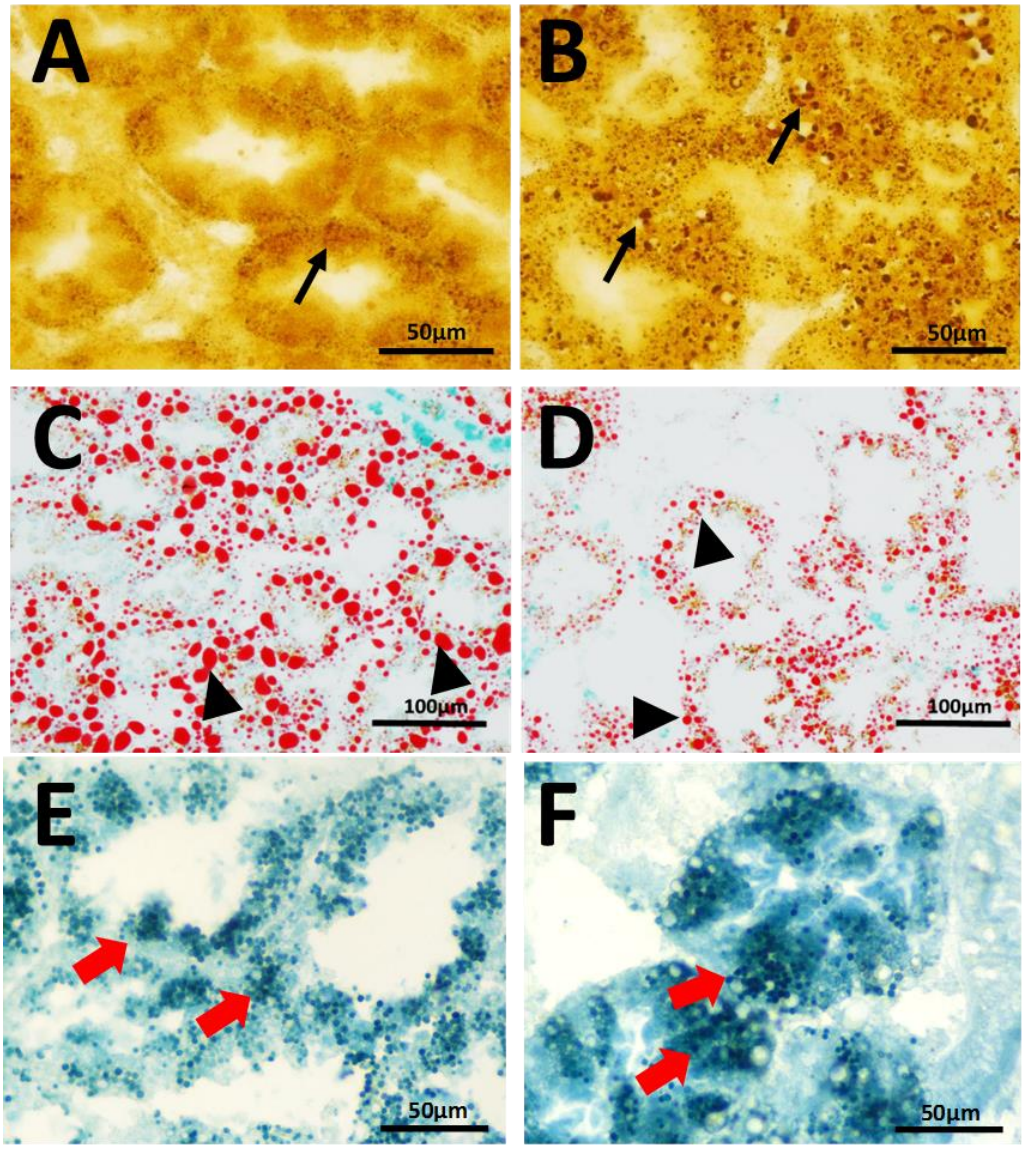
Erantzun lisosomikoak sarritan erabiltzen dira efektu-biomarkatzaile gisa, batez ere muskuiluetan eta beste molusku bibalboetan, zeintzuetan liseri-zelulak lisosometan oso aberatsak diren (JAMP, 2003; ICES, 2012). Liseri-zelulen lisosomek dituzten funtzio nagusiez gain, irensten duten materialaren zelula barneko liseriketa (Robledo et al., 2006) eta prozesu autofagikoetan parte hartzen dute (Moore et al., 2007, 2006), paper garrantzitsua jokatzen dute ere toxikoekiko erantzunetan konposatu kutsatzaileen bahiketaren eta metaketaren bitartez (Izagirre et al., 2008; Raftopoulou and Dimitriadis, 2011; Storhaug et al., 2019). Ingurugiro estresaren aurrean erantzun lisosomikoak hiru kategoria nagusitan banatu daitezke (Marigómez eta Baybay-Villacorta, 2003; Marigómez et al., 2005b): (a) mintz lisosomikoaren egonkortasuna murriztea, (b) lisosomen tamaina handitzea eta (c) lipido neutro asegabeen metaketan aldaketak bezalako eduki lisosomalen alterazioak.

Mintz lisosomikoaren ezegonkortzea kutsatzaileen eta horien ondorioen ebaluazio integratuaren planteamenduaren barnean sartzen den oinarrizko biomarkatzaileetako bat da Ipar Ekialde Atlantikoan (ICES, 2012), oinarrizko biomarkatzaile gisa hartzen da Itsaso Baltikoko Ekintza Planean (HELCOM. , 2012) eta Mediterraneoko Ekosistemen Hurbilketan (EcAp) (UNEP/MAP, 2014). Beraz, mintzaren ezegonkortzea kutsatzaile ugariren erantzun lisosomiko nagusia da eta estres orokorraren biomarkatzaile oso fidagarria gisa kontsideratzen da biomonitorizazio ikerketetan (Domouhtsidou eta Dimitriadis, 2001; ICES, 2015).

Mintz lisosomikoaren egonkortasunaren proba (LMS, *Lysosomal Membrane Stability* ingelesez) hidrolasa lisosomikoen (normalean N-acetil-ß-hexosaminidasa) jarduera latentea frogatzean oinarritzen da eta osasun lisosomikoa ebaluatzeko arrakastaz aplikatu da Bitensky et al. (1973)-en lan aitzindaritik. Substratuaren iragazkortasuna lisosometan handitzeko behar den labilizazio denborari ingelesez labilisation period (LP) deritzo (UNEP/RAMOGE, 1999). LP-aren jaitsiera esanguratsuak deskribatu dira produktu kimiko organikoen (Krishnakumar et al., 1994; Moore et al., 2007) eta metalen (Moore et al., 2007; Izagirre et al., 2014) pean. Muskuiluetan, 20 eta 25 minututik gorako LP balioek egoera osasuntsua adierazten dute, eta <10 min-ko LP balioak, berriz, osasun asaldatua edo estres egoera larria adierazten du (Viarengo et al., 2000; Izagirre eta Marigómez, 2009;).

Mintz lisosomikoaren ezegonkortzearekin batera, handitze lisosomikoa kutsatzaileekiko erantzun biologikoak ebaluatzeko jarraipen programen jarraibideetan dago (ICES, 2012). Orokorrean, lisosomak tamainaz emendatzen dira estres-baldintzetan, lisosomen bolumen-dentsitatearen emendioan (lisosomen bolumen-dentsitate handia (Vv_{LYS}) eta azalera-bolumen-erlazio baxuan (S/V_{LYS} balioak; lisosomaren tamainaren alderantzizkoa) islatua (Marigómez et al., 2005a). Zenbait kasutan, lisosomak handitzeak lisosoma-kopurua emendatzen du (Nv_{LYS}, lisosoma gehiago liseri-zelulen zitoplasman), baina Nv_{LYS}-en murrizketak ere deskribatu dira. Bestalde, kutsatzaileekiko esposizioak fase desberdinak barne hartzen dituen erantzun korapilatsua ere sor daiteke (Marigómez eta Baybay-Villacorta, 2003): (a) handitze lisosomiko iragankorra; (b) tamaina lisosomikoaren murrizketa iragankorra; eta azkenik (c) handitze lisosomikoa epe luzeko esposizioaren ondoren. Egitura-aldaketa lisosomikoak (LSC) normalean β-glucuronidasa entzima markatzaile lisosomiko gisa erabiltzen da kriotomoan egindako liseri-guruinaren ebaketetan eta irudi-analisiaren bidez zehazten dira (Izagirre et al., 2008; Izagirre eta Marigómez, 2009) (7. A eta B Irudiak). Orokorrean, parametro lisosomiko hauen erreferentzia-balioak aldakorrak dira urtaroaren arabera, baina $Vv_{LYS} > 0{,}004\ \mu m^3/\mu m^3$ eta $S/V_{LYS} < 4$ balioak muskuiluetan osasun-egoera degradatua dagoenaren adierazgarri kontsideratu daitezke (Garmendia et al., 2010; ICES, 2012).

Eduki lisosomikoen aldaketei dagokienez, zelula barneko lipido neutroen metaketa, kutsadura kimiko organikoarekin lotutako esposizio-biomarkatzailetzat hartzen da (Marigómez eta Baybay-Villacorta, 2003). Lipido neutroen metaketa lisosomikoaren handitzeak, gehiegizko lipido-tanten autofagiarekin lotuta egon daiteke (Moore, 1988; Krishnakumar et al., 1994) edo lipofuszinak izeneko pigmentuen (kaltetuako zelulen osagai hondakin liseriezinen indikatzaile direnak) formakuntzan parte hartzen dute erradikal askeek sortutako lipidoen peroxidazioari egotzi dakioke (Donato, 1981; Halliwell eta Gutteridge, 1984). Zelula barneko lipido neutroen metaketa, liseri-guruinaren kriotomoan egindako ebakiak, Oil Red O-z tindatu ondoren, irudi-analisiaren bidez zehazten da (ORO; Culling, 1974) (7. C eta D Irudiak), lipido neutroen bolumen-dentsitate bezala, liseri-epitelioaren bolumenarekin konparatuz. (Vv_{NL}; $\mu m^3/\mu m^3$) (Gomiero et al., 2015). Lipofuszinak lipidoen peroxidazioaren azken produktutzat hartzen diren pigmentuak dira (Cheung et al., 2001). Kutsatzaileen efektuen ondorioz, lipofuszinak metatzen dira liseri-zeluletan (Viarengo et al., 1990).



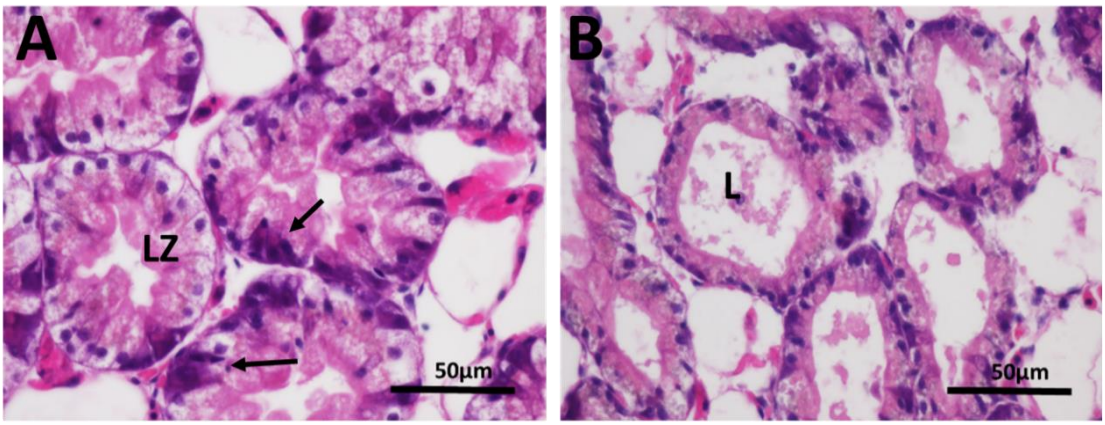
7. Irudia: Zelula mailako biomarkatzaile histokimikoetan neurtzen diren aldaketen adibideak. A,B: Lisosomen egitura aldaketak. C, D: Lipido neutroen metaketa. E,F: Liposufzina metaketa. Gezi beltzak: Lisosomak. Triangeluak: Lipido neutro tantak. Gezi gorrial: Lipofuszina granuloak.

Lipofuszinaren metaketa areagotu egin da PAH-en pean mantendutako muskuiluen liseri-zeluletan laborategiko nahiz zelai-baldintzetan (Krishnakumar et al., 1994). Lipofuszinen metaketa kriotomoan egindako liseri-guruin ebaketetan irudi-analisiaren bidez zehazten da Schmorl-en erreakzioarekin tindatu ondoren (Pearse, 1972) (7. E eta F Irudiak), lipofuszinen bolumen-dentsitatea liseri-epitelioaren bolumenarekin konparatuz (Vv_{LPF}; $\mu m^3/ \mu m^3$).

Ehun mailako biomarkatzaileak

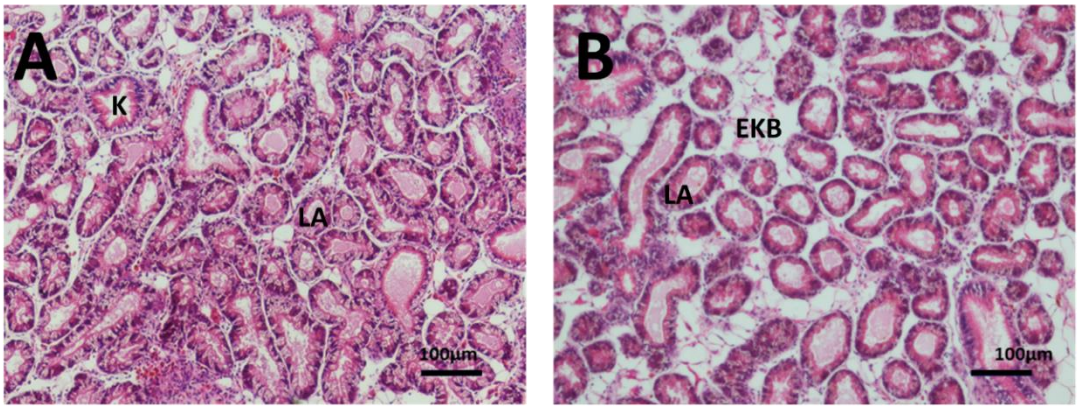
Liseri-guruinaren epitelioaren zelula-moten osaera eta albeoloen egitura-aldaketak tresna garrantzitsuak dira ingurumen-osasunaren ebaluazioan (Zaldibar et al., 2007, ICES, 2012). Eskuarki, ehun-mailako biomarkatzaile hauek hematoxilina eta eosinaz tindatutako liseri-guruin parafina laginen ebakietan prozedura estereologiko bidez zehazten dira (Garmendia et al., 2011; ICES, 2012).

Aurretik aipatu den moduan, baldintza fisiologiko normaletan liseri-zelulak zelula basofiloak baino ugariagoak dira, baina estres egoera desberdinetan, zelula basofiloen proportzio erlatiboa handitu egiten da (Soto et al., 2002; Zaldibar et al., 2007). Zelula basofiloen proportzio erlatiboaren igoera kutsaduraren efektuekin lotutako liseri-zelulen aldaketa degeneratiboekin lotu ohi da (Marigomez et al., 1990 ; ICES, 2012) (8. A eta B Irudiak). Liseri-epitelioen zelula motaren konposizioaren alterazio hauek elikagaien liseriketan eta xenobiotikoen metabolismo eta metaketan asaldurak eragin ditzake (Marigómez et al., 1998). Aldaketa hauek hasieran zelula basofiloen proliferazioari egotzi zitzaien (Lowe eta Clarke, 1989; Marigomez et al., 1990), baina beranduago nagusiki liseri-zelulen galera eta zelula basofilikoen hipertrofiaren ondoriozkoa dela proposatu zen (Zaldibar et al., 2007). Erantzun azkarra, induzigarria eta itzulgarria da, zelula basofiloen bolumen dentsitatearen arabera (Vv_{BAS}) neur daitekeena. Kutsatzaileen eraginpean egon ondoren, Vv_{BAS} 0,12 $\mu m^3/ \mu m^3$ gainditu dezake *Mytilus galloprovincialis* muskuiluetan (Garmendia et al., 2010; Marigómez et al., 2006).



8. Irudia: Muskuilu baten liseri-albeoloen liseri-ehunaren prestakin histologikoa. A: Liseri-ehun erlatiboki lodia. B: Liseri-ehun erlatiboki mehea. LZ: Liseri-zelula. L: Lumena. Geziak: Zelula basofilikoak.

Moluskuetan kutsatzaileen ondorioz hobekien dokumentatutako ehun alterazioa liseri-guruinaren epitelioaren itxurazko atrofia edo "mehetzea" da (Kim et al., 2006; ICES, 2012) (8. A eta B Irudiak). Muskuiluen liseri-guruina oso dinamikoa eta plastikoa da. Liseri-albeoloen morfologiak aldaketa handiak jasaten ditu prozesu fisiologiko arruntetan ere hau da, liseriketaren faseetan zehar (Langton, 1975).



9. Irudia: Muskuiluen liseri-guruinean ematen diren osotasunaren aldaketak bi gertakin histologikoetan. A: Ehun konektibo besikular interstizial gutxi duen liseri-guruinaren ebaketa. B: Ehun konektibo besikular interstizial emendatua duen liseri-guruinaren ebaketa. K: Konduktua. LA: Liseri-albeoloa. EKB: Ehun konektibo besikularra.

Fase digestibo arrunten alterazioak ingurumen faktoreei egotzi dakizkieke, hala nola, elikagaien eskuragarritasuna, estres osmotikoa edo termikoa (Winstead, 1995). Horrez gain, askotan deskribatu da kutsatzaileen eraginpean dauden moluskuek liseri-guruinen epitelioetan masa galera garbia erakusten dutela, epitelioaren mehetze anormala sortzen duena eta azkenik atrofia edo liseri-ehunen galera dakarrena (Garmendia et al., 2010; Rocha et al., 2016). Epitelioaren argaltzeak eta atrofiak ingurumen baldintza estresagarrien aurrean erantzun ez-espezifikoa, induzigarria eta

berreskuragarria da, zeina atrofia indizearen bidez (Kim et al., 2006) eta ehun konektibo eta ehun digestiboen arteko proportzioaren (CTD) bidez (9. A eta B Irudiak) neurtu daitezke (Garmendia et al., 2010,2011).

Biomarkatzaileetan eragin dezaketen faktoreak

Kasu askotan, biomarkatzaileen erantzunetan eragiten duten ingurumen faktoreen ezagutza ezak edo nahaste faktoreen existentziak (adibidez, elikagaien eskuragarritasuna eta kalitatea, adina edo ugalketa-zikloa) interpretazio okerrak eragin ditzake kutsatzaile mailak eta biomarkatzaileen erantzunak (Beyer et al., 2017; González-Fernández et al., 2016). Laborategiko baldintzetan, **elikagaien hornikuntzaren aldaketek** (González-Fernández et al., 2015) edo **ugaltze-faseek** eragin esanguratsua izan dute biomarkatzaile molekularren erantzunetan eta haien interpretazioan (González-Fernández et al., 2017). Faktore hauek lotura estua erakusten dute, izan ere, **hazkuntza-tasak** eta ugalketa-baldintzak bezalako faktoreek **aldakortasun geografiko** nabarmena erakutsi zuten laginketa guneen artean elikagaien eskuragarritasun desberdintasunei lotuta (González-Fernández et al., 2016).

Mytilus edulis muskuiluak hedapen zabala du eskualde boreo-epeletan, Ipar-Pazifikoan, Ipar-Atlantiko eta Erdi-Atlantikoan, Ozeano Artikoraino (Bayne eta Bayne, 1976). Ozeano Atlantikoaren iparraldekoenetan bizi diren espezie honetako banakoek izoztutako baldintzetan 8 hilabetez bizirik irauten dutela ikusi da (Bayne eta Bayne, 1976), hala ere, haien hazkuntza eta ugalketa **tenperaturaren menpekoak** dira (Berge et al., 2005; Storhaug et al., 2019). *M. edulis* espezie dioikoa da, banakoak gutxi gorabehera urte bat edo bi ondoren ugaltzeko prest daude (Bayne eta Bayne, 1976; Beyer et al., 2017). Muskuiluaren lehen sexu heltzearen hasiera tamainarekin baino adinarekin eta hazkuntza tasarekin lotuta dagoela frogatu da (Sprung, 1983). Ikerketek oskolaren luzeraren hazkundeak eta ehun bigunen hazkundeak aldakuntza independentea dutela frogatu dute (Handå et al., 2011). Norvegiako kostaldeko hainbat tokitan bildutako muskuiluetan maskorraren luzera **latitudearen** arabera aldakorra dela frogatu da, hazkuntza tasak hegoaldetik iparraldera txikiagotuz (Handå et al., 2011). Ehun bigunen hazkuntza tasak ugalketa-zikloarekin eta eskuragarri dauden elikagaien

kantitatearekin eta kalitatearekin lotuta daude (Handå et al., 2011). Gainera, **airetango esposizioa** muskuiluen hazkuntza tasetan negatiboki eragiten duen beste faktore bat da itsasarteko indibiduoetan. Marearteko eremuan aurkitzen diren indibiduoek baldintza desberdinak jasaten dituzte marea azpiko muskuiluekin alderatuta, eta itsasgoran bakarrik elikatzen dira (Sprung, 1983). Banakoak sexualki helduak direnean, barneko (nutrienteen erabilgarritasuna eta hormonen presentzia) eta kanpoko (uraren tenperatura, gazitasuna, airearekiko esposizioa eta elikagaien erabilgarritasuna) faktoreen konbinazioak ugalketaren hasiera pizten dute, arrautzak eta espermatozoideak beren folikuluetatik askatzen dira eta gonoduktutik eta sifoi exhalantetik uretara igarotzen dira (Bayne eta Bayne, 1976; Sprung, 1983). Muskuiluen ehun desberdinetako lipido edukiaren eta ugalketaren aldakuntzak aurkitu dira Norvegiako Itsasoan eta Itsaso Zurian **urtarokotasuna**rekin eta geografiarekin erlazionatuta, kutsatzaileen sentikortasunean eragina izan dezaketenak, baldintza klimatiko eta hidrologikoen desberdintasunak faktore eragile gisa nabarmenduz (Fokina et al., 2018). Biomarkatzaileen erantzunen urtaroen aldakortasuna aztertua izan da *Mytilus galloprovincialis* eta *Mytilus edulis* muskuiluetan (Aarab et al., 2011; Nahrgang et al., 2013; Storhaug et al., 2019). Biomarkatzaileen erantzunetan desberdintasun geografikoak ere aurkitu ziren Ipar Iberiar penintsulako Abra estuarioko gune ezberdinetan ur epeletan lagindutako muskuiluetan (Marigómez et al., 1996), eta Artikoan bezalako ur hotzetan dauden gune ezberdinetan (Storhaug et al., 2019). Emaitza hauek guztiek argi erakusten dute tokiko ingurune-baldintzek erantzun biologikoetan eragin dezaketela. Beraz, espezie honen ugaltze-zikloaren eta hazkundearen aldaerak ez dira soilik tenperatura desberdintasunen araberakoak, elikagaien erabilgarritasunaren araberakoak baizik (Sprung, 1983; Page eta Hubbard, 1987; Stirling eta Okumuş, 1995; Thorarinsdóttir eta Gunnarsson, 2003; Beyer et al., 2017). Ondorioz, biomarkatzaileen erantzunen interpretazio okerra egon daiteke adinarekin edo ugalketa-baldintzekin erlazionatutako faktore nahasgarri potentzialen ondorioz, kutsaduraren aurkako biomarkatzaileen erantzuna gaizki interpretatzeko arriskua pairatuz (Cuevas et al., 2015, Beyer et al., 2017;). Horregatik, argi dago faktore nahasgarri horien aldakortasun naturalak biomarkatzaileen erantzunetan nola eragin dezakeen deszifratu beharra (Bignell et al., 2008; Bellas et al., 2014; Cuevas et al., 2015; Beyer et al., 2017).

Bibalbioen histopatologia: kutsaduraren indikatzaile eta faktore nahasgarri

Ingurumen-kutsatzaile kimikoak, hala nola metalak, hidrokarburo aromatikoak, koloratzaileak, hidrokarburo kloratuak, fenolak, nitrosoaminak, petrolio gordina, eta kutsatutako sedimentuak ornogabeetan gaixotasunak eragiten dituzte, neoplasiak barne (Gardner, 1993). Kutsatzaile horien ondorioak hiru aldagairen araberakoak dira: (a) ehun bakoitzaren zaurgarritasuna; b) produktu kimiko mota; eta (c) haien kontzentrazioa (Sparks, 1993); funtsean asaldura biokimikoak sortzen dituzte, prozesu metaboliko zelularretan disfuntzioak eragiten dituztenak eta, azkenean, antolakuntza maila biologikoa konplexuagoetan ageriko bihurtzen diren aldaketa morfologiko/fisiologikoetan islatzen dira eta gaixotasun gisa identifikatzen dira (Sindermann, 1993). Hala, alterazio patologikoak konplexutasun biologiko baxuetan ematen diren asalduren islapena direla esan daiteke (Moore eta Simpson, 1992). Aldaketa histopatologikoei buruzko ezagutza, ezinbestekoa da inguruneko estres-eragileek populazio mailan duten efektu subletalen papera ulertzeko (Stentiford eta Feist, 2005).

Patologiak, prozesu biologiko naturaletan ematen diren gaixotasunen eta disfuntzioen azterketa gisa defini daiteke, itsas organismoen osasun-egoera orokorraren adierazle sentikor, erabilgarri eta potentzial gisa erabil daitezkeenak kutsaduraren kudeaketarentzako ezinbestekoa izan daitekeena. Moluskuek parasitoak eta mota askotako gaixotasunak jasaten dituzte (Kent et al., 1989; Kim et al., 1998), eta, kasu batzuetan, parasito eta gaixotasun hauek osasunean eta ugalkortasunean eragin handia izan dezakete (Coustau et al., 1991; Pérez Camacho et al., 1997). Mota ezberdineko estres-eragileak, hala nola elikagaien eskuragarritasun baxua, uraren tenperatura, gazitasuna, marea biziak eta batez ere kutsatzaileak parasito eta gaixotasunekiko suszeptibilitatea areagotu dezakete, eta horrek organismoaren osasun-egoera murrizten du (Laird, 1961). Estres-eragileek organismoen infekzioekiko erresistentzia aldatzen dute. Bereziki, estres kimikoak eta termikoak ezkutuan dauden infekzioak aktibatzen dituztela ezagutzen da, patogeno fakultatiboen jarduera suspertu eta ostalariaren erresistentzia murrizten dutela (Sindermann, 1993). Gainera,

ingurumenaren eta ostalari-parasito sistemaren arteko elkarrekintzak konplexuak dira eta ez da erraza diren bezala interpretatzen (Kennedy et al., 1995; Lafferty, 1997).

Biologikoki gaixotasun garrantzitsuenetako bat kutsatzaileek sortu dezaketen efektu kartzinogenoa da. Hala ere, neoplasiarekin nahas daitezkeen produktu kimikoekin lotutako hainbat fenomeno zelular eta beste lesio batzuk daude, besteak beste (a) hantura, akutua zein kronikoa; (b) ugaritze zelularra zauriak konpontzerakoan; eta (c) erantzun hiperplasikoak. Jakinarazi da sedimentuetan eta uretan dauden petrokimikoek eta petrolio konposatuek neoplasmen sorrera estimula ditzatekela (Sindermann, 1979). Peters (1988)-ek sarkoma hedatuak ikusi zituen petrolioarekin kutsatutako sedimentuetan bizi ziren bibalbioetan. Mix et al. (1981) eta Mix (1982) jakinarazi zuten muskuiluetan PAH-en ehun-kontzentrazioen eta neoplasien arteko korrelazioa dagoela.

Era berean, Reinisch et al. (1984) txirletan PCB-ak neoplasia hemozitikoaren prebalentziaren emendioan inplikatuta egon daitezkeela iradoki zuten. Kutsatzaile organikoen efektu subletalei dagokienez, hainbat lan daude petrolio gordinaren efektu kaltegarri baina ez-letalen inguruan (Gilfillan et al., 1977). Couch (1985)-ek ostren liseri-guruinaren epitelioaren atrofiak kutsatzaileen kontzentrazio maila altuen indikatzaile izan zitekeela iradoki zuen, kausa/ondorio erlaziorik ezarri ez bazen ere. Egiturazko aldaketa gehigarriak deskribatu dira ur kutsatuetako itsas bibalbioetan. Seiler eta Morse (1988)-ek txirlen giltzurruneko zelula birxurgatzaile eta jariatzaileak kutsaduraren eragina islatu zezaketela ikusi zuten, granulozitoma kopuru handiagoa zekarrena.

Parasitoek hainbat efektu kaltegarri sortzen dituzte ostalariengan banako mailan eta, kasu batzuetan, ostalari-populazioen tamainan eta dinamikan eragin ikaragarria ere sortzen dute. Haien prebalentzia eta infekzio intentsitateak oso aldakorrak izaten dira populazioen artean (Caceres-Martinez et al., 1996; Powell et al., 1999). NOAA-ren 'Mussel Watch' egoera eta joeren datuek infekzio-intentsitate oso aldakorrak erakusten dituzte, nahiz eta prebalentziak eskualdeko jarraitasun nabarmena izan parasito arrunt askotan (Powell et al., 1999). Gainera, jakina da kutsadura kronikoko baldintzetan bizi ziren bibalbioak parasitoen infestazioekiko erresistentzia gutxiagotu erakusten dutela eremu garbietan bizi direnekin konparatzerakoan (Sunila, 1987; Svärdh eta Johannesson, 2002). Winstead eta Couch (1988)-ek ostrak (*Crassostrea virginica*) n-

nitrosodietilamina (DENA) kontzentrazio altuen pean mantendu zituzten 20ºC-tan, *Perkinsus marinus* parasitoa esposatutako ostretan maila altuagoetara biderkatu zela kontroleko ostretan baino deskribatu zuten, hilkortasun handiagoa eraginez. *C. virginica*-ren maila baxuko (< 3,0 ppb) DDT, toxafeno eta paratioi esposizio kroniko baxuak mizelio fungiko baten infekzioa eragin zuen, kontroleko ostrak inbaditu ez zituena (Lowe et al., 1971).

Hilkortasun masiboen kasu askotan, seguruenik patogeno infekzioso eta ingurumeneko estresaren konbinazioa da azkenean organismoak eskala handian hiltzen dituena (Farley eta Durfort, 1987). *Bonamia ostrea* eta *Marteilia refringens* bezalako parasito batzuek eragin larriak eta hilkortasun handiak eragin ditzake bibalbioen populazioaetan (Perkins eta Wolf, 1976; Villalba et al., 1993). Izan ere, gaixotasunak kudeatzeko estrategia sendoak behar dira molusku populazioen egonkortasuna babesteko.

Muskuiluak infektatzen dituzten organismo patogenoen artean hurrengoak dira ezagunenetarikoak:

Mikroorganismoek sortutako gaixotasunak

Rickettsia/*Chlamidia* antzeko organismoak.

Rickettsia/*Chlamydia* antzeko organismoak (RLO/CLO) (10. A Irudia), "*Prokaryotic Inclusion Bodies*" (PIB) bezala ere ezagutzen dira, hauek parasito arruntak dira uretako animalia askotan, arrainetan (Zachary eta Paperna, 1977), moluskuetan (Wu, 2003) zein krustazeotan (Bonami eta Pappalardo, 1980; Johnson, 1983). Bakterio Gram-negatibo hauek, zelula barneko patogeno moduan deskribatzen dira eta, ostalarien zelulen barnean ugaltzen dira (Chen et al., 2000) eta oro har, inklusio-gorputz basofilo moduan behatzen dira mikroskopioan (Renault eta Cochennec, 1994; Wen et al., 1993), nahiz eta inklusio basofilikoak aurkezten dituen ostalari berean inklusio intrazitoplasmiko eosinofiloak ere ikusi dira (Wen et al., 1993). Sarritan pikortsuak dira, ia homogeneoak eta modu irregularrean kokatuta daude mantu, zakatz eta liseri-albeoloen epitelio-zeluletan, mantuaren eta zakatzen ehun konektiboetako zeluletan, eta, kasu horietan, ehun konektibo besikularrean (Wu eta Pan, 1999). Infektatutako

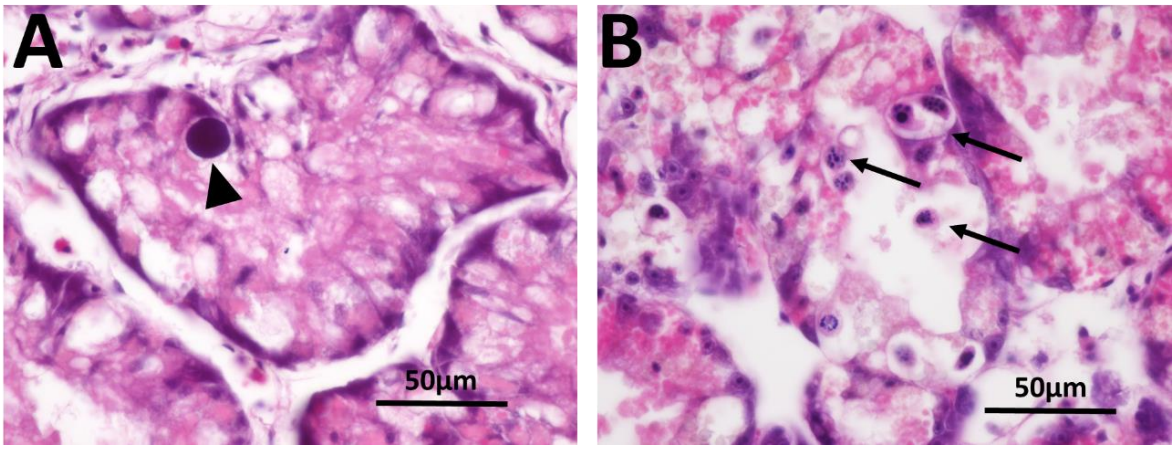
zelulak azkenean lisia pairatzen dute, RLO/CLO-ak albeoloen lumenera askatuz (Meyers, 1979), non desintegrazioa gertatzen den infekzioaren azken faseetan (Todd et al., 1976). Ostalariaren hanturazko erantzunak prokariotoen infekzioaren aurrean ez dira detektatu ere egin suntsiketa tubularra agerikoa denean (Villalba et al., 1997). Intentsitate baxuko infekzioak, oro har, ez dira patogenoak, baina parasito hauen kolonia handiek zelula egituraren suntsipena, barne endekapena, bakuolizazioa, hantura eta organulu zelularren suntsipena eragin dezakete (Sun eta Wu, 2004). Organismo hauek ez dute zelula ostalaria hiltzen ekintza toxikoz edo interferentzia metaboliko/fisiologikoz, baizik eta zelulen suntsipenaren bidez (Romero et al., 2000). Normalean, prebalentziak baxuak dira, gutxitan % 20-tik gorakoak (Powell et al., 1999), ohikoena izanik albeoloen atrofia maila baxua duten organismoetan, eta horrek ziurrenik ehun osasuntsua beharrezkoa dela infekziorako (Powell et al., 1999). Hala ere, noizean behin % 100 prebalentziak ere deskribatu dira (Powell et al., 1999) Mexikoko Golkoan hidrokarburo aromatikoen maila altuetara esposatutako muskuiluetan, funtzio immune fisiologiko garrantzitsuak mekanikoki hondatzen direla iradokitzen duena. Muskuiluarentzat kaltegarriak direnik ikusi ez arren, hilkortasun garrantzitsuak deskribatu dira krustazeoetan (Bower et al., 1996), bieira eta txirletan (Villalba et al., 1999), eta *Haliotis*-etan (Moore et al., 2000).

Protistek sortutako gaixotasunak

Ciliophora (zelula barneko ziliatuak).

Bibalbioekin lotuta dauden ziliatu gehienak, flagelatuak bezala, ziurrenik kaltegabeko komentsalistak dira. 150 ziliatu espezie baino gehiago aurkitu dira itsas bibalbioen mantuaren barrunbean, zakatzetan eta dibertikulu digestiboetan. Ziliatu horietako gehienek normalean komentsal gisa jardun arren, patogeno bihur daitezke beraien kopurua oso altua denean, ostalariaren egoera fisiologikoa arriskuan dagoenean edo ingurumen estres eragileei ezin zaienean behar bezala aurre egin (Lauckner, 1983). Muskuiluen X Protozooa (MPX) (10. B Irudia) eta zelula barneko beste ziliatuak albeolo digestiboetan kokatzen dira, batez ere liseri-zelulen barruan (Villalba et al., 1997). Parasito intrazitoplasmatiko hauek Rhynchodid-antzeko Phyllopharynge ziliatu gisa hartu ziren Bower et al. (1994)-engatik. Ardatz formako gorputza dute, makronukleo

polimorfoa eta basofiloarekin eta hainbat mikronukleo esferiko ere aurkezten dituzte. Ziliatuak zelula gurasoaren ardatz laburrean zeharkako fisioaren bidez ugaltzen dira normalean, horrela bi banako berdin osatuz. Nahiz eta infekzioek ostalariaren erantzunik ez sortu ziliatuak dituzten zelula ostalarien handitzea baino, infekzio larriek liseri-epitelioen haustura eragin dezakete.



10. Irudia: Parasito desberdinen infekzioak dituzten muskuiluen gertakin histologikoak. A: RLO/CLO-ren presentzia. B: MPX ziliatuaren presentzia. Triangelua: RLO/CLO, Geziak: MPX ziliatuak.

Metazooek sortutako gaixotasunak

Trematoda

Esporozisto infekzioen (Bucephalidae antzeko organismoak, adibidez) (11. A Irudia) hasierako faseak gonada ehunetan burutzen dituzte, gero esporozistoak zakatz, mantu eta beste ehunetara hedatzen dira (Cheng eta Burton, 1965). Garapen-etapa desberdinak germen-bolatik esporozistoaren barruan ia helduak dauden zerkariak bereiz daitezke (Canzonier, 1972). Infekzio larrietan, ugaltze organoen atrofia osoa (kastrazio parasitikoa, esterilizazioa eta gametogenesia ezabatzea eragiten duena) beha daiteke paraleloan beste ehunen kaltearekin batera, batez ere hodien eta senuen oklusioa, desplazamendu edo konpresioaren ondorioz (Canzonier, 1972). Kasu gehienetan, ez da ostalariaren erantzun nabarmenik gertatzen, nahiz eta noizean behin hemozitoen infiltrazioak gerta daitezkeen parasitoen inguruan. Orokorrean ostalari

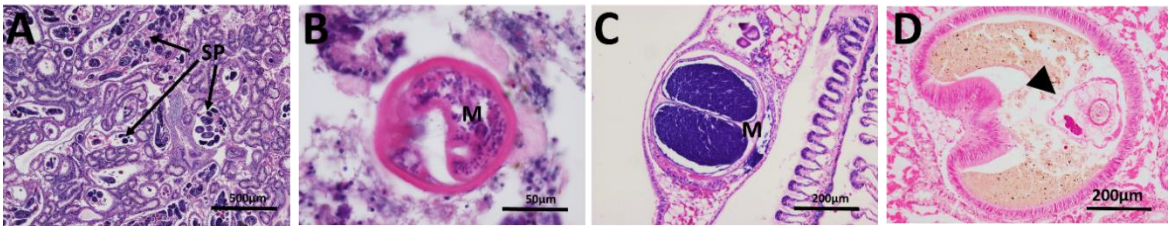
populazioen dinamikan eragin nabarmena izateko ez dira agertzen nahiko prebalentzia altuetan. Gainera, baldintza normaletan, esporozistoen infestazioei zuzenean egotzitako hilkortasunak ez dira esangarriak (Jensen eta Mouritsen, 1992).

Metazerkaria infekzioak (10. B eta C Irudiak) ehun konektibo besikularrean eta oinean ematen dira batez ere. Infekzioa urtean zehar aldatzen da (Hughes eta Answer, 1982), ziurrenik ostalariaren ugaltze-zikloarekin lotura duenak, baina aldaketa horiek ez dira berdinak populazio guztietan ingurumen desberdintasunengatik (uraren tenperatura, korronteak, gazitasuna…) (Svärdh, 1999). Metazerkaariaz infektatutako ostalarietan ikusitako efektu batzuk (Cremonte et al., 2005; Cremonte eta Ituarte, 2003): (a) hemolinfaren osagaien alterazioak, (b) hazkundearen murrizketa tasa; (c) balboak ixteko ahultasuna; d) bisu ekoizpenaren murrizpena; e) ostalariaren portaeraren aldaketak; eta f) ahultasun orokorra eta hilkortasuna. Gainera, hanturekin, parasitoaren enkapsulazio hemozitikoekin eta granulozitomekin lotuta daude metazerkaria infekzioak (Lee et al., 2001). Oroar, metazerkariak eragindako patologia aldakorra da eta parasitoaren eta ostalari-espeziearen araberakoa da.

Copepoda (*Mytilicola sp.*)

Mytilicola intestinalis kopedodo parasito bat da (10. D Irudia), bibalbio sorta zabal baten hesteetan bizi dena, muskuiluak barne. Hainbat ikerketek kopepodo hauek heste funtzional bat ez dutela behar bizimodu endoparasitiko bat mantentzeko ondorioztatu dute (Durfort, 1971). Hala ere, Durfort (1977)-ek *Mytilicola*-ren hestean hiru zelula-mota deskribatu zituen eta Gresty (1992)-k *Mytilicola*-ren liseri-hodia aktiboki parte hartzen duela mantenugaien barnerapenean ondorioztatu zuen. Hiru zelula motak hauek dira: (a) R-zelulak, erretikulu endoplasmiko leuna dutenak; (b) F-zelulak, erretikulu endoplasmatiko pikortsua dutenak, mitokondrio eta besikula ugarikoak; eta (c) B-zelulak, bakuolo oso handiak dituztenak gorputz trinko txikiz eta bakuoloz inguratuta (Arnaud et al., 1978). *Mytilicola sp*.-ren infekzio prebalentzia eta intentsitatea ostalariaren tamainarekin erlazionatuta dago, muskuilu handietan txikietan baino kopepodo gehiago agertzen dilerarik, Olivas-Valdez eta Cáceres-Martínez (2002)-ek *Pseudomyticola spinosus* kopepodoarentzat *Mytilus galloprovincialis* muskuiluan, Goater eta Weber (1996)-ek *Mytilicola orientalis* kopepodoarentzat *Mytilus trossulus* muskuiluan, eta Gee and Davey (1986)-k *Mytilicola*

intestinalis kopepodo gorriarentzat *Mytilus edulis* muskuluan deskribatu bezala. Honek infektatutako muskuilu ostalarietan hainbat ondorio sortzen ditu: (a) gonaden tamaina murriztua eta ugalketa atzeratua (Williams, 1969); (b) oskol-hazkunde murriztua (Theisen, 1987); (c) bisu ahuldua (Brienne, 1964); (d) iragazketa eraginkortasun murriztua (Meyer eta Mann, 1951); (e) hesteetako epitelioaren higadura eta metaplasia (Villalba et al., 1997). Hala ere, ez da efektu nabarmenik aurkitu populazio mailan (Davey et al., 1978), organismo mailan (Gresty, 1992) edo maila zelularrean (Moore et al., 1978) egindako beste lan batzuetan. Gainera, ez da ostalariaren hilkortasunaren frogarik deskribatu kopepodo kopuru handiak dituzten muskuiluetan (Olivas-Valdez eta Cáceres-Martínez, 2002). Horrela, nahiz eta kopepodo honek kalteak eragiten dituen ez du bere ostalaria hiltzen.



11. Irudia: Parasito desberdinen infekzioak dituzten muskuiluen gertakin histologikoak. A: Trematodoen esporozistoak. B: Trematodo metazerkaria bat liseri-guruinean. C: Trematodo metazerkaria mantuan. D: Mytilicola sp. bat liseri-traktuan. SP: Esporozistoa, M: Metazerkaria, Triangelua: *Mytilicola sp*.

Erantzun immunitarioak bibalbioen histopatologian

Parasitoez gain analisi histopatologiko egokia egiteko alterazio patologikoen agerpena ikertzea beharrezkoa da eta modu egokian burutzeko bibalboen sistema immunologikoaren ezagutza ezinbestekoa da.

Laburki, molusku bibalboen sistema immunologikoa zelulen bitartekaritza mekanismo eta mekanismo humoralez osatuta dago. Moluskuek sistema immunologikoa dute, baina ez dute memoria immunea, beraz ez dute antigorputzik sortzen. Parasitoek eta beste alterazio batzuen presentzian moluskuen hemozitoak erreakzionatzen dutenean lau defentsa mekanismo bereiz daitezke (Cheng, 1981): (a) hemozitosia, infekzioarekiko lehen erantzuna dena eta zirkulatzen duten hemozitoen kopurua handitzea dakar (b) fagozitosia, infekzioaren hurrengo urratsa (kimiotaxia, ezagupena, atxikimendua, endozitosia eta suntsipena) (c) enkapsulatzea, organismo edo partikula inbaditzailea handiegia denean gertatzen dena (d) nakresazioa, mantuaren eta

oskolaren arteko espazioa inbaditzen duten parasitoen edo kanpoko materialaren inguruan nakarra jartzen denean gertatzen dena (adibidez, *Mytilus* perlak sortzea *Gymnophallus bursicola* parasito trematodoaren inbasioari erantzunez).

Muskuiluetan, hemozitoak bi mota nagusitan sailkatu dira: granulozitoak (10-20 µm-ko diametroa) eta agranulozitoak edo hialinozitoak (4-6 µm-ko diametroa) (Cajaraville eta Pal, 1995). Hemozitoak gorputzean zehar mugitzen dira hemolinfan eta hainbat barneratu daitezke ehunetan haien mugimendu propioaren bidez (diapedesia; (Cheng, 1984). Prozesu honek xenobiotikoak hemolinfa edo ehunetatik kanporatzen dituen desintoxikazio-sistema espezifiko bat errepresentatzen du, eta horrela odolean eta ehunetan kontzentrazioa mantentzen maila toxikoen azpitik da (Marigomez et al., 1990; Soto et al., 1996). Infiltrazio hemozitikoa ehunen kaltearen ondoren konponketa prozesu bat izan arren (DesVoigne eta Sparks, 1968), efektu patologikoak izan daitezke erantzun immune horren erantzule (Couch, 1984; Lee et al., 2001).

Ehun konektiboaren aldaketa patologikoak oso anitzak izan daitezke. Hanturazko erantzun ez-espezifikoen agerraldia goseteak eta kumatzeak eragindako estresarekin lotzen dira, baina baita kimikoki eragindako estresarekin ere (Couch et al., 1979; Lowe et al., 1972). Moluskuek hidrokarburo aromatikoak, pestizida kloratuak eta metalak bezalako kutsatzaileetara esposatuta egoterakoan baldintza nahiko garbietan bizi diren moluskuak baino maizago tamaina ezberdineko granulozitomak aurkezten dituzte ehun ezberdinetan (Svärdh, 1999; Wolfe, 1992). Petrolio gordinaren eta PAH-en eraginpean *Mya truncata* eta *Mytilus edulis*-en liseri-guruinean granulozitomen intzidentzia altuagoak deskribatu dira (Neff et al., 1987; Wolfe, 1992), baita industria-isuriek eta metal astunek kutsatutako guneetako organismoetan ere (Wedderburn et al., 2000). Gainera, Villalba et al. (1997) granulozitomek eragindako dibertikuluen egitura suntsitze zabala ikusi zuten. Era berean, infiltrazio hemozitiko handiak aurkitu ziren *Mya arenaria*-ren ehun konektiboetan Tampa badian oso urbanizatutako estuario batera transplantatzerakoan (Nasci et al., 1999). Era berean, hemozitoen bolumen-dentsitatearen igoera nabarmena erregistratu zen *Littorina littorea*-ren liseri-guruinaren ehun konektibo interstizialean Cd-aren eraginpean egotearen ondorioz (Marigómez et al., 1990). Zelula arreen agregatuak ere izan dira xenobiotikoek eragindako estresaren adierazletzat hartzen dira (Zaroogian eta Norwood, 2002). Zelula arreak ugariagoak,

handiagoak eta koloreztatuagoak daude gune kutsatuetako organismoetan toki garbikoetan baino (Zaroogian eta Yevich, 1993).

Orokorrean, molusku bibalboen alterazio patologikoak ez dira arrainen histopatologia bezain beste erabiltzen kutsaduraren nazioarteko jarraipen programetan, dosi-erantzunen harremanak eta beste faktore biotiko eta abiotikoen patologietan duten influentziaren inguruan ezagumendu falta dagoelako (Au, 2004).

Ikerketaren testuingurua: H2020 GRACE proiektua

Doktorego tesi hau Europar Batasuneko 2020 Horizon GRACE proiektuaren barruan kokatua dago: “Integrated oil spill response actions and environment effects” (2016-2019; http://www.grace-oil-project.eu).

Itsaso Baltikoak itsas trafikoaren presio handia jasotzen duenez (Lavrova et al., 2014) eta Ipar Atlantikoko eskualde artikoek aurkitu gabeko petrolio eta gas erreserba kopuru nabarmena dutenez (Gautier et al., 2009), mehatxatutako gune gisa kontsideratu daitezke. Beraz, petrolio isuriak ugaritzeko arriskua emendatzen ari da eta haien ondorio ekologiko eta sozioekonomiko arriskutsuen azterketa garrantzitsua da. Gainera, ekosistemak Artikoak oso zaurgarriak dira petrolio-isurien aurrean, beren ingurumen-baldintza bereziengatik (itsasoko uraren tenperatura baxua eta, nabarmenagoa dena, izotz-estalkiaren presentzia) eta urruntasunagatik. Ezaugarri hauek isuritako produktuen WAF-en konposizio kimikoa alda dezakete eta itsas biotarekiko toxikotasuna areagotu (Nordam et al., 2017) eta Olio-isurien ondoren garbiketa operazioak oztopatzen duten faktore nagusiak izan daitezke. Horrela, GRACE proiektuaren helburu nagusia klima hotzean petrolio-isuriek duten eraginari buruzko ikuspegi holistiko bat lortzea zen, eta itsasoko petrolio-isuriei aurre egiteko hainbat teknologien eraginkortasuna eta ingurumen-ondorioak ebaluatzea, izotzez infestatutako eremuetan, Ozeano Atlantikoaren iparraldean. eta Itsaso Baltikoan (Jørgensen et al., 2019). Era berean, GRACE-k petrolio-isurien ingurumen- eta sozioekonomia-inpaktuei aurre egiteko ezagutza garatu eta eskaintzea du helburu, erabakiak hartzen laguntzeko tresna aplikagarri gisa (Jørgensen et al., 2019). Helburu

nagusia lortzeko, proiektua bost lan paketetan banatu zen, horietako bakoitza gai zehatz batean zentratuta:

- Petrolio-isurien detekzioa, jarraipena, patua eta banaketa
- Petrolioaren biodegradazioa eta bioerremediazioa
- Petrolioaren inpaktuak biotan biomarkatzaileak eta arrisku ekologikoen ebaluazioa erabiliz
- Kostalde artikoko uretan petrolio isuriari aurre egitea: eraginkortasuna eta ingurumen-ondorioak
- Erabakiak hartzeko ingurumenaren onurarako analisi estrategiko bat (sNEBA) garatu eta abian jartzea

Doktorego tesi hau ingurumen-baldintza ezberdinetan gertatzen diren petrolio-isurien inpaktu biologikoak eta petrolio-isurien aurrean eskualde hotz hauetan erabiltzen diren erantzun estrategien eragina (adibidez, sakabanaketa kimikoa eta in situ errekuntza) ebaluatzera bideratutako lan-paketean sartu zen. Lan hau bereziki garrantzitsua izan zen GRACE proiektu europarrean hirugarren lan paketearen barruan. Izan ere, proiektuko ikerketa gune geografikoetako (Ipar Ozeano Atalntikoa, Ozeano Artikoa eta Itsaso Baltikoa) muskuiluen biomarkatzaileen maila basalei buruzko eta faktore nahasgarrien efektuen deskribapenari buruzko informazioa oso urria da eta parametro hauk, esperimentazio toxikologikorako eta ingurugiroan eman daitezkeen hondamendien inpaktua ebaluatzeko beharrezkoa den informazio kritikoa eskaintzen dute.

Lan-pakete ezberdinetan egindako lana elkarrekin oso lotuta dago eta txosten ugaritan eskuragarri dago (http://www.grace-oil-project.eu). Azkenik, GRACE proiektuak lortutako eraginak hainbat izan ziren eta dira (Jørgensen et al., 2019) petrolio-kutsadurak eta erantzun metodologiek itsas ingurunean, kostaldeko ekonomietan eta komunitateetan dituzten eragin negatiboak arintzen laguntzean dutenak. Baldintza desberdinetan petrolio-isuriei erantzuteko estrategiarako erabakiak hartzen laguntzeko tresna hobeak eskuragarri egitea, besteak beste. Komunitate zientifikoaren eta gobernuen arteko komunikazioa hobetzea, eta petrolioari erantzuteko ekipoak eta jarraipen zerbitzuak ekoizten dituzten enpresei ezagutza eskaintzea dira baita ere proiektu honetan bultzatu diren beste helburu batzuk.

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2 AUZIAREN EGOERA, HIPOTESIA ETA HELBURUAK

AUZIAREN EGOERA

Kostaldeko ekosistemen osasun egoera ebaluatzeko tresnarik erabilienak azkeneko urteetan organismo zentineletan oinarritutako jarraipen programak dira. Azken hamarkadetan paradigma aldaketa graduala gertatu da, non organismoek ehunetan dituzten kutsatzaileen analisi kimikoa soilik egitetik, organismo zentinela hauek kutsatzaileen aurrean erakusten dituzten erantzun biologikoak erabiltzera pasa den. Neurgarriak diren erantzun biologiko hauek biomarkatzaile bezala ezagutzen dira, eta analisi kimikoek ematen duten informazioaz gain, informazio osagarri garrantzitsua eskuratu daiteke jarraipen prometan aplikatzen badira, hau da, organismo zentinelak pairatzen duten estres kimiko edo/eta naturalaren efektu biologikoak ulertzea ahalbidetzen dute.

Muskuiluak (*Mytilus sp.*) biojarraipen prometan organismo erabilienetarikoak dira aurkezten dituzten ezaugarri biologikoengatik: sesilak eta iragazleak dira, beraz ingurunearen egoeraren adierazle egokiak dira. Gainera kutsadura maila erlatiboki altuetan bizitzeko gai dira, baina biomarkatzaileak aplikatzeko egokiak dira kutsadurarekiko sentikorrak direlako ere. Honela, hainbat eremu geografikoetan ikerketa asko gauzatu dira muskuiluen biomarkatzaile sorta zabala erabiliz, ondorioz, erantzun hauen oinarrizko balioak eta ingurugiroko estres sortzaileen aurrean biomarkatzaileen balio maximoak era egokian definituz. Honek garrantzia kritikoa du, faktore natural eta fisiologikoek pisu handia baitaukate biomarkatzaileetan, ekosistemaren osasun egoeraren ebaluazio oker bat ekar zezakeena. Biomarkatzaileetan efektua eduki dezaketen faktore natural edo fisiologikoei faktore nahasgarriak deritze.

Ipar Ozeano Atlantikoak, Ozeano Artikoak eta Itsaso Baltikoak aurkezten dituzten muturreko baldintza naturalengatik faktore nahasgarrien efektuak are eta garrantzitsuagoak suerta daitezke, gainera, petrolioari lotutako jarduerek bertan sortzen duten ingurugiro arriskuak zaintza berezia behar duten guneak bihurtzen dituzte hauek. Eremu geografiko horietan faktore nahasgarrien potentziala ez dago guztiz ikertuta, orain arte egin diren ikerketatan aukeratutako biomarkatzaile bateriek

orokorrean ez dituztelako ehun mailako biomarkatzaileak eta histopatologia barneratu. Ehun mailako biomarkatzaileak eta analisi histopatologikoak funtsezko informazioa ematen dute organismoaren prozesu fisiologikoei eta estres maila orokorrei buruz, konplexutasun biologiko baxuagoko erantzun biologikoek ematen ez duten moduan. Gainera, analisi histopatologiko zehatz batek, parasito eta patologiek biomarkatzaileen erantzunetan estresik sortzen ari diren konfirmatzea ahalbidetzen digu, beste faktore nahasgarri potentzial baten efektuak determinatuz. Ehun mailako biomarktzaileak, ikerketa eremuan dagoeneko erabili diren beste biomarkatzaileekin (zelula mailakoak, biokimikoak…) konbinatzen badira, lehenengoak ematen duten informazio berria dagoeneko eskuragarri dagoen informazioarekin konparatu daiteke erreferentzia moduan, honela lagindutako muskuiluen osasun egoeraren ebaluazio zehatza gauzatuz. Hori dela eta, ekosistema desberdinen osasun egoera era holistikoan aztertzeko muskuilu zentineletan antolakuntza maila desberdinetan kuantifikatutako biomarkatzaile bateriaren erantzun gaitasuna argitzea ezinbestekoa suertatzen da, eta honek lekuen arteko konparaketa eta estrapolazioa ahalbidetuko lituzke, kutsaduraren jarraipen programen eta esperimentu toxikologikoen diseinu eraginkorragoetan itzuli zitekeena.

HIPOTESIA

Muturreko ingurumen baldintzak dituzten gune artiko eta subartikoetako *Mytilus sp.* zentinelen osasun egoeraren ebaluazio holistikoa eta konparaketa integratzaile eta zehatzak burutzea ahalbidetzeko, zelula eta ehun mailako biomarkatzaileen oinarri balioak finkatzea eta aldakortasun naturala identifikatzea beharrezkoa da egokiak diren kutsaduraren jarraipen programak aurrera eramateko.

HELBURUAK

Hipotesi hau enpirikoki frogatu ahal izateko hurrengo helburu hauek ezarri ziren:

1- Urtaroei lotutako ingurumen-faktoreek aukeratutako biomarkatzaile bateria batean izan ditzaketen efektuak deskribatzea, Itsaso Baltikoko ingurumen baldintza desberdinak dituzten bi azpieskualdeetan, erreferentziazko datuak ezartzeko etorkizunean biojarraipen programetan erabiliak izateko (1. Atala).

2- Kokapen subartiko eta artiko baten arteko aldakortasun naturalak (latitudearekin erlazionatutako ingurumen-faktoreak eta bildutako animalien adina) zelula eta ehun mailako biomarkatzaile hautatuen baterian duen influentzia identifikatzea, kutsaduraren aurrean erantzuteko duen gaitasuna determinatzeko, 2016ko udazken hasieran leku erlatiboki ez kutsatu eta kutsatuetan lehen hurbilketa moduan bildutako bi tamainetako (adinetako) muskuiluetan (2. Atala).

3- Ipar Ozeano Atlantiko eta Ozeano Artikoan tarte latitudinal zabal batean zehar lagindutako muskuiluetan osasun egoera kaltetua eta erantzun biologiko alteratuen bidez kutsadurarekiko erantzunak maskaratu dezaketen aldaketa histopatologikoak identifikatzea (3. Atala).

4- Aldakortasun naturalak (latitudearekin erlazionatutako ingurumen-faktoreak eta bildutako animalien adina) zelula eta ehun mailako biomarkatzaile hautatuen baterian duen eragina identifikatzea, kutsaduraren aurrean erantzuteko duen gaitasuna determinatzeko, 2017ko uda amaieran ozeano Ipar Atlantikoan eta Artikoan definitutako tarte latitudinal zabal batean leku ez kutsatu eta kutsatuetan lagindutako bi tamainetako muskuiluetan (4. Atala).

3 RESULTS

1. Atala: Urtaroei lotutako aldagai ekologikoen eragina biomarkatzaileen oinarri balioetan Itsaso Baltikoko bi eskualdetako muskuiluetan (*Mytilus trossulus*)

Argitalpena: Benito, D., Ahvo, A., Nuutinen, J., Bilbao, D., Saenz, J., Etxebarria, N., ... & Soto, M. (2019). Influence of season-depending ecological variables on biomarker baseline levels in mussels (*Mytilus trossulus*) from two Baltic Sea subregions. Science of the Total Environment, 689, 1087-1103.

Kongresuak:

CICTA 2018: General stress biomarkers in mussels (Mytilus trossulus) collected in three different seasons from two regions of the Baltic Sea. Denis Benito, Dennis Bilbao, Nestor Etxebarria, Ionan Marigómez, Xabier Lekube, Urtzi Izagirre, Beñat Zaldibar, Manu Soto, Aino Ahvo, Kari K. Lehtonen. Oral Presentation

SETAC 2019: effect of season-depending ecological variables on The responsiveness of a battery of biomarkers in Mussels (mytilus trossulus) from two localities in the Baltic sea. D. Benito, A. Ahvo, D. Bilbao, J. Saenz, N. Etxebarria, X.L. Iturrioz, U. Izagirre, K.K. Lehtonen, I. Marigomez, B. Zaldibar, M. Soto.

ABSTRACT

For reliable mussel monitoring programmes based on biomarkers, regionally relevant reference values and their natural variability need to be known. The Baltic Sea exhibits high inter-regional and seasonal variability in physical factors such as salinity, temperature and primary production. The aim of this pilot study is to depict the effects of season-related environmental factors in a selected battery of biomarkers in two environmentally different subregions of the Baltic Sea to help establishing reference data for biochemical, cellular and tissue-level biomarkers. In order to achieve that, mussels were collected from reference sites in Kiel (Germany) and Tvärminne (Finland) during three seasons: summer and autumn 2016, and spring 2017. Finally, in order to characterize the ecological situation, analysis of the chemical tissue burden was performed and chlorophyll-*a* and particulate organic carbon concentration and temperature changes were analyzed at each sampling locality using satellite remote sensing images. An integrated biomarker response index was performed to summarize the biomarker responses of each locality and season. The biochemical endpoints showed seasonal variability regulated by temperature, food supply and reproductive cycle, while among the cellular endpoints only lipofuscin accumulation and lysosomal structural changes showed slight seasonal variation. Seasonal changes in tissue level biomarkers were observed only at the northern Baltic Sea site Tvärminne, dictated by the demanding energetic trade-off caused by reproduction. In conclusion, the characterization of the ecological variables and physico-chemical conditions at each site, is crucial to perform a reliable assessment of the effects of a hypothetical pollution scenario in the Baltic Sea. Moreover, reference levels of biomarkers and their responses to natural environmental conditions must be established.

LABURPENA

Muskuiluetan oinarritutako biomarkatzaileak erabiltzen dituzten jarraipen programa fidagarriak burutzeko, eskualdeko erreferentzia balioak eta haien aldakortasun naturala ezagutu behar dira. Itsaso Baltikoko faktore fisikoak oso aldakorrak dira urtaro eta lekuaren arabera, hala nola gazitasuna, tenperatura eta ekoizpen primarioa. Lan pilotu honen helburua biomarkatzaile bateria batean urtaroarekin lotutako ingurumen-faktoreen efektuak irudikatzea da Baltikoko bi eskualdetan, biomarkatzaile biokimiko, zelular eta ehun mailako erreferentzia datuak ezartzen laguntzeko. Hori lortzeko, Kiel (Alemania) eta Tvärminne (Finlandia) erreferentziazko guneetatik muskuiluak bildu ziren hiru sasoietan: 2016-ko udan eta udazkenean, eta 2017-ko udaberrian. Azkenik, egoera ekologikoa ezaugarritzeko, ehunen konposatu kimikoen kontzentrazioaren azterketa egin zen eta a-klorofila eta karbono organiko partikulatuen kontzentrazioa eta tenperatura aldaketak aztertu ziren laginketa leku bakoitzean satelite bidezko teledetekzio irudiak erabiliz. Biomarkatzaileen erantzun indize integratua egin zen, toki eta urtaro bakoitzeko biomarkatzaileen erantzunak laburtzeko. Biomarkatzaile biokimikoek tenperaturaren, elikagai-eskuragarritasunaren eta ugaltze zikloaren arabera erregulatutako urtaro aldakortasuna erakutsi zuten, eta zelula mailako biomarkatzailen artean, lipofuszina metaketek eta lisosomen egitura aldaketek urtaroko aldakuntza txikia soilik. Ehun mailako biomarkatzaileen urtaro aldaketak Tvärminne-n (Itsaso Baltikoko iparraldeko gunean) bakarrik ikusi ziren, ugaltzeak eragindako eskaera energetiko zorrotzak aginduta. Ondorioz, gune bakoitzeko aldagai ekologikoen eta baldintza fisiko-kimikoen karakterizazioa funtsezkoa da Itsaso Baltikoan kutsadura eszenatoki hipotetiko baten ondorioen ebaluazio fidagarria egiteko. Gainera, biomarkatzaileen erreferentzia mailak eta ingurumen baldintza naturalen erantzunak ezarri behar dira.

SARRERA

Kutsatzaileen mailak gora egin du ingurune itsastarretan giza jardueren ondorioz. Uraren eta sedimentuen kalitatearen pobretze honek itsas organismoetan aldaketak sor ditzake. Beraz, giza jarduerei lotutako kutsatzaileen arriskuaren ebaluazioa egitea beharrezkoa da. Ingurumen osasunaren ebaluazioa ezin da analisi kimikoan soilik oinarrituta egin, ez baitu informaziorik ematen kutsatzaileek izaki bizidunetan eragiten dituzten ondorio kaltegarriei buruz. Hori dela eta, kutsatzaileek eragiten dituzten efektu biologikoen neurketak garrantzi handia hartu du ebaluazio hauetarako (Cajaraville et al., 2000, Rementeria et al., 2016, Benito et al., 2017).

Muskuiluak, batez ere *Mytilus* generokoak, kostaldeko eta estuarioetako ekosistemen osasuna aztertzeko helburua duten kutsaduraren jarraipen programetan gehien erabiltzen diren organismo zentinelak dira (Cajaraville et al., 2000, Marigómez eta Baybay-Villacorta, 2003, Nasci et al., 2002, Brenner et al., 2014). Muskuiluek estres sortzaile kimikoekiko esposizioa jasaten dute eta era anitzetan erantzuten dute, biomarkatzaileen bidez neurtu daitezkeenak (Garmendia et al., 2011).

Azken hamarkadetan, organismo urtarretan biomarkatzaileen aplikazioa kutsaduraren eragin biologikoen adierazle gisa oso erabilia izan da. Biomarkatzaileak tresna baliotsuak dira ingurumen osasuna ebaluatzeko. Dena den, estres kimiko hipotetiko baten eraginaren pean neurtutako erantzun biologikoen interpretazio zuzena lortzeko, beharrezkoa da biomarkatzaileen aldakortasun naturalaren balio tarteak ezagutzea baldintza ekologiko desberdinetan, hala nola tenperatura, gazitasuna eta elikagaien erabilgarritasuna (Leiniö eta Lehtonen, 2005, Nahrgang et al., 2013, Beyer et al., 2017, Storhaug et al., 2018).

Itsaso Baltikoak ingurumen faktoreen aldakortasun (geografiko eta urtarokotasunezkoa) izugarria aurkezten du. Esaterako, tenperatura urtaroaren arabera (0-tik 20°C-ra) eta gazitasuna latitudearen arabera (18-20 psu inguruko gazitasuna hego-mendebaldean eta 3-4 oso iparraldeko zatietan) aldatzen dira. Aldakortasun handi horrek espezieen egokitzapen fisiologikoak eskatzen ditu, eta beraz, biomarkatzaile gisa neurtutako erantzun biologikoetan urtaroen aldaketen ondorioak espero daitezke.

Lan honetan aukeratutako Itsaso Baltikoko bi azterketa eremuetan lehenago egindako ikerketetan (Turja et al., 2015) kutsatzaile maila baxuak deskribatu ziren eta, gainera, ingurumen-baldintza desberdinak aurkezten dituzte. Hori dela eta, arrazoizkoa da urtaroak biotan duen eragina bi eremuen artean aldatzea. Udaberriko alga loraldi intentsiboaren garapena disolbatutako mantenugai ez-organikoen kontzentrazioen beherakadaren eta klorofila-kontzentrazioa handitzearen ondorioz egiaztatu daiteke. Uraren tenperatura 0 eta 20° C artekoa izan ohi da urtean zehar, eta Finlandiako Golkoan izotz estalkia garatu ohi da urtarrilaren amaieratik apirilaren hasierara bitartean, nahiz eta izotzaren iraupena urtero aldatzen den eguraldi-baldintzen arabera. Izotza urtzean, fitoplanktonaren ekoizpena azkar handitzen da eta Finlandiako golkoko udaberriko loraldia, oro har, maiatzaren hasieran iristen da maximora (Leiniö eta Lehtonen, 2005). Golkoko erdialdean eta iparraldean zianobakterio loratze toxiko masiboak gertatzen dira udaro, normalean uztailaren hasieran/erdialdean edo abuztuaren hasieran (Lehtonen et al., 2003). Mendebaldeko Itsaso Baltikoan eragin handia dute hondotik sartzen den Ipar Itsasoko ur gazi emariak (batez ere neguan) eta gainazalean zehar ematen den Baltikoko ur gazi isurketa etengabeak (otsailetik udara arte handituz). Itsaso Baltikoaren zati honetan, fitoplanktonaren loraldiaren hazkuntza aldi nagusia otsailaren erdialdetik martxoaren erdialdera edo martxoaren erdialdetik apirilaren hasierara bitartekoa da. Desberdintasun hauek urteko aldaketa meteorologiko eta hidrografikoekin lotuta daude. Uraren nahasketa bertikala handitzen denean (iraila inguruan), hondoko uretatik elikagai bermineralizatuen goranzko garraioa dela eta, udazkeneko loraldia gerta daiteke (Wasmund et al., 2008).

Itsas kutsaduraren jarraipen programek biomarkatzaile molekularrak, zelularrak eta ehun mailakoak gero eta gehiago konbinatzen dituzte, kutsatzaileen eragin biologikoak ebaluatzeko (Marigómez et al., 2013). Hala eta guztiz ere, biomarkatzaileen erantzun gaitasunean nahaste faktore intrintsekoek eragina izan dezakete, hala nola organismo behaleen ugaltze egoerak. Beraz, biomarkatzaileen erantzun batzuen aldakortasuna aurkitzea espeziearen urteko ziklo fisiologikoan naturala da (Sheehan eta Power, 1999). Itsaso Baltikoan tenperaturaren eta elikagai ugaritasunaren urtaroetako aldakortasun handiak (Kautsky, 1982) muskuiluen ugalketa-zikloan beste hainbat itsasotan baino urtarokotason nabarmenagoa sortzen du (Ortiz-Zarragoitia et al., 2011;

Azpeitia et al., 2017). Hori dela eta, ikerketa pilotu honen helburu nagusia urtaroarekin lotutako ingurumen faktoreen eragina irudikatzea da aukeratutako biomarkatzaile bateria batean, ekologikoki desberdinak diren Itsaso Baltikoko bi eskualdeetan erreferentziazko datuak ezartzeko. Horretarako, Finlandiako mendebaldeko Golkotik eta Itsaso Baltikoko mendebaldetik muskuiluak bildu ziren urtaro desberdinetan aldakortasun naturala aztertzeko, konplexutasun biologiko maila ezberdinetako erantzunak adierazten dituzten biomarkatzaile bateria erabiliz. Hasteko, laginketa guneak karakterizatzeko a klorofila (Chl-a) eta karbono organiko partikulatuen (POC) kontzentrazioak eta tenperatura aldaketak aztertu ziren laginketa leku bakoitzean. Muskuiluen ehun bigunetako kutsatzaile kontzentrazioa (metalak eta hidrokarburo aromatiko poliziklikoak [PAH] barne) ere neurtu zen. Muskuiluen erantzun biologikoei dagokienez, zelula adipogranularren (ADG) dentsitatean eta gametoen garapen egoeran aldaketak zehaztu ziren parametro laguntzaile gisa. Maila biokimikoan, liseri-guruinean katalasa (CAT) eta glutation S-transferasaren jarduerak (GST) eta lipidoen peroxidazioa (LPO) neurtu ziren, eta azetilkolinesterasaren jarduera (AChE) zakatzetan. Zelula mailan, muskuiluen liseri-zeluletan mintz lisosomikoen egonkortasuna (LMS), egitura aldaketa lisosomikoak (LSC) eta lipofuszinen (Vv_{LPF}) eta lipido neutroen (Vv_{NL}) bolumen dentsitateak kuantifikatu ziren. Zelula basofiloen bolumen dentsitatea (Vv_{BAS}), liseri-albeoloen epitelioaren atrofia eta liseri-ehun eta ehun konektiboaren arteko erlazioa (CTD) kalkulatu ziren ehun mailako erantzunak zehazteko. Horrez gain, alterazio histopatologikoak aztertu ziren liseri-guruinean, mantuan eta zakatzetan.

MATERIAL ETA METODOAK

Ingurugiro faktoreen urrutiko detekzioa

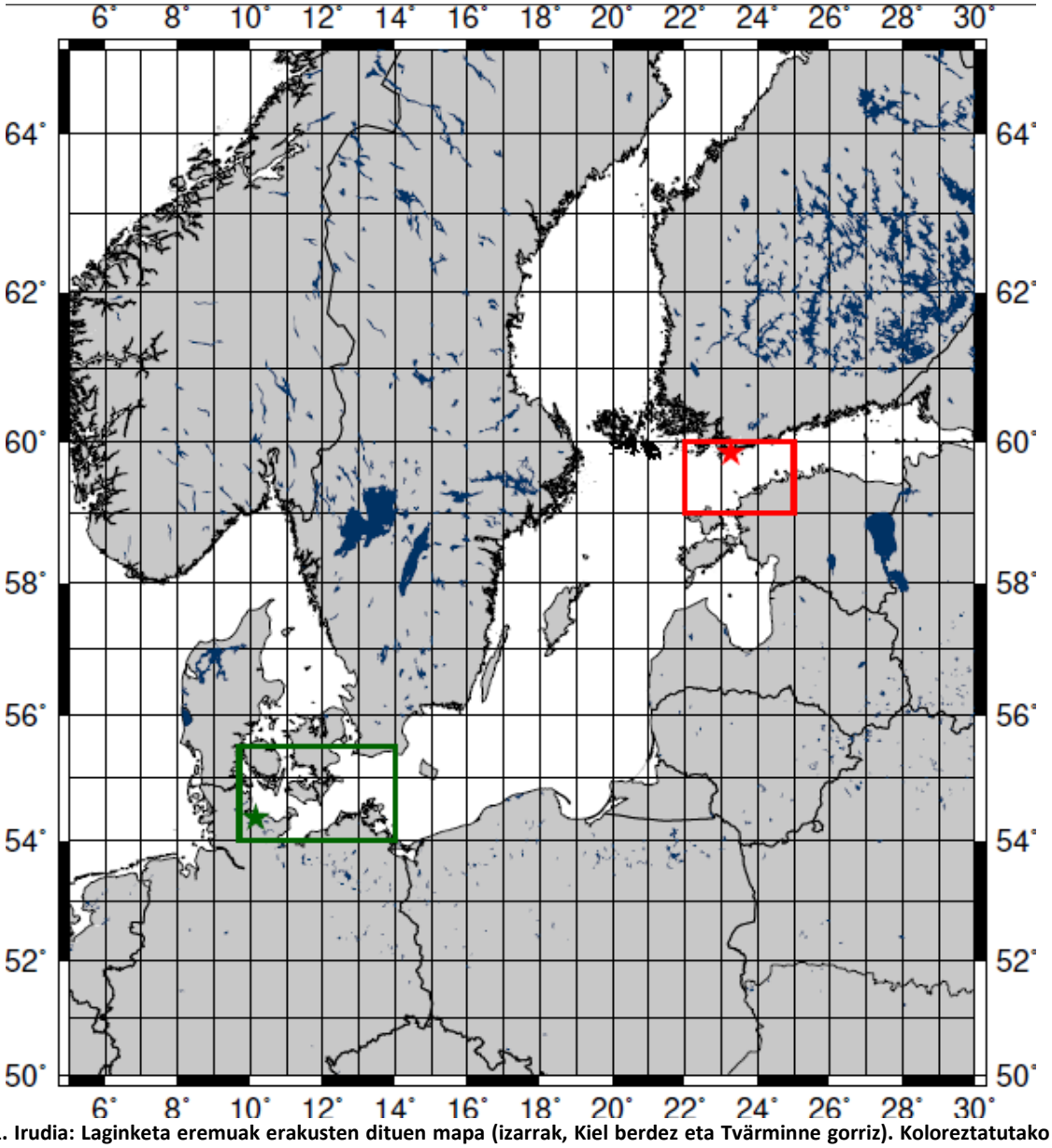
Laginketa egunean eta aurreko egunetan itsas ingurunearen eskualde-eskalako ebaluazioa lortzeko, 4 km-ko bereizmeneko “Mapped Level-3 MODIS” eguneroko datuak (Aqua eta Terra sateliteak) NASAko “Ocean Color” serbidoreetatik deskargatu ziren 2002ko urriaren 1-tik 2018-ko urriaren 1-erarte. Deskargatutako datuak chl-a pigmentu fotosintetikoaren (CHLOR_A, mg m-3, Hu et al., 2012) eta karbono organiko partikulatuaren (POC, mg m-3, Stramski et al., 2008.) kontzentrazioei dagozkie, fitoplankton biomasaren eta produkzio primario ozeanikoaren deskribatzaileak direnak,

hurrenez hurren (Honjo et al., 2008; Raateoja et al., 2018), eta baita eguneko eta gaueko itsaso gainazaleko tenperaturari ere (SST, Celsius, Kilpatrick et al., 2015) 11 µm-ko uhin-luzeran.

Ipar latitudeetan falta diren datuen kopurua oso handia da, hodei estaldurak erradiazio infragorriaren hedapen atmosferikoan eragiten duelako. Itsas ingurunearen eskualdeko egoera irudikatzeko erabilgarri dagoen pixel-kopurua handitzeko, gaueko eta eguneko sateliteen SST eremuen batez bestekoa egin da, egunez edo gauez bakarrik zentratu beharrean (Kozlov et al., 2014), aurreko ikerketek frogatu baitute SST datuen fidagarritasuna handia dela eremu honetan (She et al., 2007). Satelitetik eratorritako eguneroko datuekin laginketa guneen inguruko koadroetan batez besteko espazialak egin dira (1. irudia). SSTren kasuan, Aqua eta Terra sateliteen eguneko eta gaueko kutxaren batez besteko datuen batez besteko bakarra erabili da, 1. irudian irudikatzen diren koadroetan eguneko banakako datuak emanez. Sateliteek berreskuratutako aldagai guztietarako eguneko urtaro ziklo leunak egin dira Egun Juliano berari dagozkion eguneroko balioen multzoari dagozkion medianak lortuz, Aqua eta Terra sateliteen prozesatutako 17 urteetako datuekin (2002ko urriaren 1etik 2018ko urriaren 1era). Kasu honetan batez bestekoaren ordez mediana erabiltzea justifikatuta dago aldagai batzuetan (bereziki CHLOR_A eta POC) laginketa aldiaren hasieran edo amaieran agertzen den zarata saihesteko. Eguneroko medianaren araberako urtaro-zikloak nahiko irregularrak dira, eta 11 eguneko batez besteko baten bidez leundu dira, batez bestekoaren kalkuluan parte hartzen duten egunen % 60 gutxienez egonez gero bakarrik kalkulatzen dena. Modu honetan, sasoi ziklo leunduaren definizioan outlier-ek duten eragina gutxienekoa da. Ondoren, urtaro ziklo leunduak erabili dira CHLOR_A, POC eta SSTren eguneroko anomaliak definitzeko. Urtaroko ziklo leunduez gain, exekutatzen diren batez bestekoak (zazpi egun) erabiltzen dira satelite bidez eratorritako aldagai horien eguneroko balioak irudikatzeko 2. irudian.

Laginketa

Muskuiluen (*Mytilus trossulus*) bilketa hiru laginketa sasoitan egin zen (2017/04/04 Finlandian, 2017/03/17 Alemanian, 2016/07/07 Finlandian, 2016/08/17 Alemanian eta 2016/10/26 Finlandian, 2016/11/08 Alemanian), Itsaso Baltikoko iparraldean (Finlandiako Golkoan; Tvärminne-ko Estazio Zoologikoan, Helsinkiko Unibertsitatearen parte dena, Hanko penintsulan 59°50'45.39"N 23°15'41.81"E) eta hego-mendebaldean (Kiel Fjord 54°22'11.24"N 10°9'17.70"E) (1. Irudia).



1. Irudia: Laginketa eremuak erakusten dituen mapa (izarrak, Kiel berdez eta Tvärminne gorriz). Koloreztatutako laukiek batez besteko espazialak egin diren eremuak irudikatzen dituzte.

Itsaso Baltikoko marea eza dela eta, ikerketa helburuetarako muskuiluak urpekaritza bidez bildu ziren 8 metro inguruko sakoneran. Murgilketa egin eta berehala muskuiluak sailkatu ziren eta 2,5-3,0 cm-ko luzera zuten 100 muskuilu guneko urarekin jarri ziren, ontzi termoisolatuetan. Muskuiluak laborategira eraman ziren eta giroko tenperaturan zegoen uretan mantendu ziren hurrengo egunera arte, disekzio egunera arte, alegia. Disekzio bakoitzean, mantua, zakatzak eta liseri-guruina barneratzen zituzten ebaketa transbertsalak egin ziren 20 muskuilutan analisi histopatologikoa eta ehun-mailako biomarkatzaileen analisia egiteko. 30 muskuiluren zakatzak eta liseri-guruina disekzionatu eta nitrogeno likidoan izoztu ziren, biomarkatzaile histokimiko eta biokimikoak aztertzeko. Muskuilu osoen lagin osagarriak izoztu ziren ehun bigunetako kutsatzaileak neurtzeko.

Laginen prozesamendua

Ebaketa transbertsalak % 4 formaldehidotan (itsasoko uretan diluitua) fixatu ziren, etanol bainu serie batean deshidratatu eta parafinatan murgildu Leica ASP3005 ehun prozesadorea erabiliz eta 5 µm-tako lodierako xaflatan ebaki ziren Leica RM2125RTS mikrotomo batean analisi histopatologikorako eta ehun-mailako biomarkatzaileak aztertzeko. Liseri-guruin bakoitza izoztuta gorde zen -80 °C-tan, eta gero 8 µm-tako zatitan ebaki ziren CM3050s Leica kriotomo bat baliatuz analisi histokimikorako. Izoztutako zakatzak eta liseri-guruinak biomarkatzaile biokimikoetarako erabili ziren.

Ehunetako PAH edukia

30 muskuilu taldekatuen ehun bigun laginak homogeneizatu ziren eta 5-10 g hartu laginketa gune eta urtaro bakoitzeko PAH-en estrakziorako. Barneko (Fluoro-PAH-ak, Chiron) eta etekineko PAH estandarrak homogenatuari gehitu zitzaizkien. Bost ml ur eta 10 ml etilazetato gehitu ziren eta laginak minutu batez irabiatu. 4 g $MgSO_4$z eta 2 g NaClz osatutako gatz nahasketa gehitu zen. Laginak berriro minutu batez irabiatu eta gero 10 minutuz zentrifugatu ziren. Etilazetatoaren estraktuaren gainnadantearen bost ml hartu ziren eta 200 µl isooktano gehitu zitzaizkion erauzketari. Etilazetatoa nitrogeno

fluxuaren azpian lurrundu zen eta 1 ml hexano gehitu zen. Estraktua beira artilea, Na_2SO_4 eta silizea zituen zutabe batean araztu zen. Hexanoan zeuden PAH konposatuak, hexano/diklorometanoarekin eluitu ziren (3:1, v/v). Eluzioa egin ondoren 0,5 ml isooktano gehitu zen disolbatzaileen gordetzaile gisa eta disolbatzailea nitrogeno-fluxu leun baten azpian lurrundu zen 0,5 ml-ko azken bolumena lortu arte. Hamar µl estandar injekzio gehitu ziren (PAH deuteratuak, Dr. Ehrenstorfer) eta lagina Thermo GC-MS/MS batekin (Trace 1310 GC Ultra gas kromatografoa eta TSQ Quantum XLS ultramasa espektrometroa) aztertu zen. Neurketak hautatutako erreakzioen monitorizazioa (SRM) moduarekin egin ziren. PAH konposatuen identifikazioa bi ohiko ioi fragmentu hautatuz egin da. Pisu freskoen unitateak pisu lehorreko unitate bihurtu ziren, muskuiluen ehun bigunen ur ehunekoan oinarritutako zuzenketa-faktore bat erabiliz (Potrykus et al., 2003).

Ehunetako aztarna metalen edukia

Laginketa gune eta urtaro bakoitzeko 20 muskuiluren taldekapena liofilizatu, homogeneizatu, pisatu (± 0,0001 g) eta Teflon estaldura zuten ontzietara transferitu ziren mikrouhin labean liseritzeko. Ontzi bakoitzean 2 ml HNO_3 (%69, Tracepur, Merck), 2 ml H_2O_2 (%30 Fluka, p.a.) eta 2 ml MilliQ ur (Millipore Element) gehitu ziren. Ontziak berogailu pasiboko zilindroan muntatu eta Teflon tapoiekin itxi ziren.

Mikrouhin labearen berotzeko programa aurrez optimizatuta zegoen eta potentzia programa bat jarraitu zuen (Anton Paar, Multiwave Pro). Lehen urratsa 600 W-ra igotzea izan zen 5 minututan eta hor 5 minutu gehiago mantentzea. Ondoren, potentzia 1000 W-ra arte handitu zen 5 minututan eta 5 minutuz mantendu zen. Azkenik, potentzia itzali (0 W) eta labea presurizatu egin zen lagina hozteko (15 min). Programa honetan ohiko presio eta tenperatura altuenak 20 bar eta 190^oC ingurukoak ziren eta ehun biologikoen liseriketa oso-osorik burutu zen. Errotoreak 8 laginen liseriketa ahalbidetzen zuen aldi berean, baina horietako bi sistematikoki “blank”-en analisirako gorde ziren.

Estraktua xiringa batera transferitu eta PVDF 0,45 µm-ko disko iragazkien bidez iragazi zen. Teflon estaldurako ontzi eta tapoi guztiak MilliQ urarekin garbitu eta modu

berean iragazi ziren. Azken disoluzioa zehatz-mehatz pisatutako Falcon hodietan prestatu zen eta MilliQ ura gehitu zen 50 ml arte. Azken Falcon hodiak pisatu ziren disoluzioaren masa zehatza lortzeko. Azkenik, disoluzio guztiak diluitu ziren, azken azidotasuna disoluzio estandarrenarekin bat etortzeko (% <1).

Analisi elementala ICP-MS bidez (NexIon200, Perkin Elmer) egin zen. Laginak ponpa peristaltiko bat erabiliz xurgatu ziren nebulizazio sistemara (Meinhard nebulizatzailea) eta, aldi berean, lerro paralelo baten bidez, barneko disoluzio estandarren fluxu jarraitua (Ge, Sc eta Y) nahastu eta neurtu zen neurketa erroreak gainditzeko. Isotopo hauek ^{7}Li, ^{27}Al, ^{88}Sr, ^{98}Mo, ^{107}Ag, ^{120}Sn, ^{121}Sb, ^{137}Ba, ^{184}W, ^{205}Tl eta $^{206+207+208}$Pb (Barne estandarrak: Sc, In eta Bi) neurtu ziren. ^{47}Ti, ^{51}V, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{78}Se ^{111}Cd eta ^{202}Hg (Barne estandarrak Sc, Y, Ge eta In) He talka modua erabili zen. Nahiz eta lan askotan metal multzo txikiagoa hartzen den kontuan, horiek guztiak hautatu ziren balizko markatzaile multzo zabal bati aurre egiteko eta dagoeneko laborategiko ohiko prozeduretan zeudelako (Rodriguez-Iruretagoiena et al., 2016).

Zelula adipogranularren indizea

Mantuko erreserbako energia materialaren adierazle gisa, adipogranular (ADG) zelula indizeak ugalketarekin eta urtaroko aldaketa bioenergetikoekin lotutako estrategia metabolikoaren iradokizun bat eman dezake (Bignell et al., 2008). Mantuko zelula adipogranularrek glukogenoa, lipidoak eta proteinak dituzte (Danton et al., 1996), eta zelula horien dentsitatea mantuko ehun germinalaren kantitatearen alderantziz proportzionala dela frogatu da (Moukrim et al., 2008). ADG zelulen dentsitatearen ebaluazioa Bignell et al. (2008)-ek deskribatutako moduan (n=20) egin zen. Honako kalifikazio sistema erabili zen:

- 0: ez dago ADG zelularik ehun konektibo besikularraren barruan.
- 1: ADG zelulak ikus daitezke baina eskasak dira.
- 2: ADG zelulak mantuan zehar sakabanatuta agertzen dira.
- 3: ADG zelulen ugaritasuna nabarmen handitu da, baina baliteke ehun eremu batzuek erabateko konsistentzia ez edukitzea.
- 4: ADG zelulek ehun konektiboaren bolumenaren gehiengoa osatzen dute.

Gametoen garapena

Gametoen garapen etapak prestaketa histologikoetan zehaztu ziren (n=10) Ortiz-Zarragoitia et al. (2011) -ek deskribatzen duten moduan: atseden-fasea (aktibitaterik gabe edo desberdindu gabea); hasierako fase gametogenikoa (gametogenesia hasi da baina ez dago gameto heldurik); etapa gametogeniko aurreratua (gametogenesia aurrera doa eta gameto helduek eta garatzen ari diren gametoek proportzio berdina dute); heldutasun etapa (gonada guztiz heldua, folikuluak obuluz edo espermatozoidez beteta); errute fasea (gametoen igorpen aktiboa, folikulu batzuk hutsik agertzen dira); errute osteko fasea (folikulu hutsak eta gametoa hondarrak bakarrik geratzen dira).

Biomarkatzaile biokimikoak

Kokapen eta sasoi bakoitzeko 15 muskuiluren liseri-guruinak 100 mM potasio fosfato tanponean homogeneizatu ziren (pH 7,4). Homogeneizatuaren laginak hartu ziren LPO analisirako eta % 4 BHT gehitu zen peroxidazioa inhibitzeko. Gainontzeko homogeneizatua 10.000 g-tan zentrifugatu zen 20 minutuz 4°C-tan eta gainnadanteak -80°C-tan gorde ziren aztertu arte.

Kokapen eta sasoi bakoitzeko 30 muskuiluren zakatzak homogeneizatu ziren % 0,1 Triton X (pH 7,0) duen 20 mM sodio fosfato buffer-etan eta 10 000 g-tan zentrifugatu 20 minutuz 4 °C-tan. Gainnadanteak -80 °C-tan gorde ziren aztertu arte.

Entzima-jarduera guztiak 96 putzuko plaketan neurtu ziren mikroplaka irakurgailu baten bidez (TECAN Infinite 200) eta Magellan softwarearekin (TECAN)

aztertu. Liseri-guruinen laginak GST, CAT eta LPO-rako aztertu ziren, eta brankietako laginak AChErako. Proteinen kontzentrazioa Bradford (1976) metodoaren bidez zehaztu zen behi-serum albuminaren estandar batekin (Sigma A7030).

GST jarduera, hidrokarburo aromatiko poliziklikoen (PAH) eta bifenilo polikloratuen (PCB), esposizio biomarkatzailea da, bai arrainetan bai ornogabeetan (Leiniö eta Lehtonen, 2005). Klorodinitrobentzeno-glutation (CDNB-GSH) substratu konjokatuaren eraketa tasa gisa neurtu zen 340 nm-tan, Habig et al-en arabera. (1974). Erreakzioan CDNB (Sigma-Aldrich 138630) eta GSH (Sigma-Aldrich G4251) 1 mM-ko kontzentrazioak 100 mM potasio fosfato tamponean, pH 7,0, erabili ziren.

CAT jarduera, adibidez metalen esposizioak eragindako estres oxidatiboaren adierazlea da eta uretako organismoetan ikertuak izan dira (bibalbioak barne) (Leiniö eta Lehtonen, 2005). Hidrogeno peroxidoaren (H2O2, Fluka 95302) degradazio gisa neurtu zen CAT 240 nm-tan (Claiborne, 1985). Erreakzioko H2O2-ren azken kontzentrazioa 4,3 uM izan da 100 mM potasio fosfato tamponean, pH 7,0.

LPO maila oxidazio kaltearen adierazle da (Shaw et al., 2004) eta azido tiobarbiturikoarekiko substantzia erreaktibo gisa neurtu zen 535 nm-tan (Ohkawa et al., 1979). Erreakzio nahasketak 60 mM Tris-HCl zituen azido dietilentriaminepentazetikoarekin (DTPA, Sigma-Aldrich D6518), 0,24 M azido trikloroacetikoarekin (Sigma-Aldrich T6399) eta 16 mM azido 2-tiobarbiturikoarekin (Sigma-Aldrich T55000) osatu zen.

AChE jarduerak metalak, detergenteak eta alga toxinak bezalako produktu kimikoekiko duen sentikortasuna onartua da eta estres neurotoxikoa adierazten du (Leiniö eta Lehtonen, 2005). AChE jarduera azetiltiokolinaren (ACTC) hidrolisia 412 nm-an neurtuz zehaztu zen (Bocquené eta Galgani, 1998). Erreakzio-nahasketak 0,57 mM azido 5,5'-ditiobis-2-nitrobenzoiko (DTNB, Sigma D8130) eta 2,9 mM azetiltiokolina ioduro (Sigma A5751) 20 mM sodio fosfato tamponean (pH 7,0) zituen.

Zelula mailako biomarkatzaileak

LMS eta LSC estres orokorraren biomarkatzaileak dira (Marigómez et al., 2006, Izagirre et al., 2008). LMS-aren zehaztapena UNEP/RAMOGE (1999) liseri-zelulen

lisosometan hexosaminidasa (Hex) aktibitatea frogatu ondoren tindatze intentsitate maximoa sortzeko beharrezkoa den labilizazio azido tratamenduaren denboran oinarritzen da. Prestakinak (talde bakoitzeko 10 liseri-guruin) 4 °C-tan jarri ziren 30 minutuz eta gero 10 minutuz giro tenperaturan mantendu ziren tindatu aurretik. Kriotomoan egindako ebaketa serialak (10 µm) 0, 3, 5, 10, 15, 20, 25, 30, 35 eta 40 minutuko tarteetan 0,1 M zitrato tanponean (pH 4,5 % 2,5 NaCl duen pH 4,5) egon ziren 37 °C-ko ur bainuan, mintz lisosomala guztiz labilizatzeko behar den aurretratamendu denbora ezagutzeko. Tratamendu honen ondoren, ebaketak substratuaren inkubazio-mediora transferitu ziren Hex jarduera ikusteko. Inkubazio medioa 20 mg naftol AS-BI-N-acetil-bD-glukosaminidaz (Sigma, N 4006) 2,5 ml 2-metoxietanoletan disolbatuak (Merck, 859), eta 50ml bolumen izan arte 0,1 M zitrato tampona gehituta (pH 4,5). Ondoren, % 2,5 NaCl eta 3,5 g biskositate baxuko polipeptido (Sigma, P5115) duen 0,1 M zitrato-tanpoia gehitu zen, sekzio egonkortzaile gisa jarduteko. Ebaketak medio honetan 20 minutuz 37 °C-tan inkubatu ziren, disoluzio gazi batean (% 3,0 NaCl), 37 °C-tan 2 minutuz garbitu eta gero 0,1 M fosfato tamponera pasa ziren giro tenperaturan 10 minutuz (pH 7,4) 1 mg/ml diazonio Fast Violet B gatza koloratzailea zuela (Sigma, F1631). Ondoren, prestakinak azkar garbitu ziren iturriko uretan 5 minutuz, 10 minutuz fixatu ziren % 2,5 NaCl zuen Baker-en kaltzio formolean 4 °C-tan eta ur destilatutan garbitu ziren. Azkenik, prestakinak Kaiser-en glizerina gelatinan muntatu ziren. Tindatze intentsitate maximoa lortzeko beharrezkoa den labilizazio azido tratamendu denbora argi mikroskopio bidez ebaluatu zen lisosomekin lotutako erreakzio produktuaren metaketa maximo gisa (UNEP/RAMOGE, 1999). Animalia bakoitzerako lau determinazio egin ziren labilizazio azido sekuentziako ebaketa bakoitza lau segmentu gutxi gorabehera berdinetan banatuz eta dagokion segmentu multzo bakoitzean labilizazio aldia ebaluatuz. Ondoren, atal bakoitzerako batez besteko balioa atera zen, liseri-guruin bati dagokiona.

β-glukuronidasaren jarduera histokimikoa fixatu gabeko kriotomo ebaketetan (10 liseri-guruin talde bakoitzeko) frogatu zen Moore (1976)-ek deskribatu bezala. Ebaketak (8 µm) CM3050 kriotomo batean moztu ziren -25 °C-ko ganbera tenperaturan, beirazko prestakin epeletan bildu eta -40 °C-tan gorde ziren tindatu arte. Ebaketak giro tenperaturan epeldu ziren 5 minutuz eta, ondoren, 0,96 ml sodio bikarbonatotan (50

mM) disolbatutako 22,4 mg naftol AS-BI-β-D-glukuronidoz osatutako substratuaren inkubazio mediora transferitu eta 80 ml osatu ziren 0,1 M azetato tamponarekin (pH 4,5) % 2,5 NaCl eta 12 g polibinilo dituena, % 20 (w/v) kontzentrazioan koloide egonkortzaile gisa. Ebaketak 20 minutuz inkubatu ziren 37 °C-tko ur bainu batean, etengabeko agitazioarekin. Ondoren, prestakinak %2,5 NaCl-tan garbitu ziren 37 °C-tan 2 minutuz eta giro tenperaturan tindatu ziren 10 minutuz iluntasunean, 1 mg/ml fast garnet GBC 0,1 M fosfato tamponean (pH 7,4) % 2,5 NaCl-rekin. Ondoren, ebaketak Baker-en kaltzio formolean fixatu ziren (% 4 formaldehidoan, % 1 kaltzio kloruroan, % 2,5 sodio kloruroan) 10 minutuz 4 ° C-tan eta ur destilatutan garbitu. Azkenik, ebaketak astiro-astiro garbitu ziren ur destilatutan eta Kaiser-en gelatinan muntatu ziren.

Muskuiluetan liseri-zelulen lisosomen egitura kuantifikatzeko prozedura estereologiko bat aplikatu zen, irudiak aztertzeko sistema bat erabiliz. Sistema B&W-CCD bideokamerak, Leitz Laborlux argi-mikroskopioak, bideo plaka duen ordenagailuak eta BMS softwareak osatzen dute. 100× handipeneko objektiboa erabili zen. Liseri-zelulen zitoplasmatik lisosomak bereizten dituzten irudi bitarrak segmentazio prozeduraren bidez lortu ziren. Irudien analisi sistemarekin bolumen dentsitate lisosomikoa (Vv_{LYS}), gainazal bolumen erlazio lisosomikoa (S/V_{LYS}) eta zenbakizko dentsitate lisosomikoa (Nv_{LYS}) lortu ziren. Bost neurketa egin ziren liseri-guruin bakoitzeko. Formula estereologikoek zuzenketa faktore bat barne hartzen dute ebaketaren lodiera baino batez besteko diametro txikiagoa duten partikulen kasuan (Lowe et al., 1981). Laginaren tamaina lau parametroen batez besteko eta desbideratze estandarraren balioen aurretiko analisietan oinarrituta zehaztu zen, gutxienez 16.000 μm^2 baino gehiagoko laginketa eremu batean konstante mantentzea lortu zutenak (Etxeberria et al., 1994). Neurketa bakoitzean eskaneatutako liseri-zelulen azalera osoa 4.000 μm^2 ingurukoa zenez, 5 neurketa egin ziren ebaketa bakarrean (muskuilu bakoitzeko laginketa eremu osoa 20.000 μm^2).

Lipofuszinak lipidoen peroxidazioaren edo liseriketaren azken produktutzat hartzen dira (Cheung et al., 2001). Era berean, moluskuen liseri-guruinean lipofuszina-kontzentrazioaren igoera kalte oxidatiboarekin (Viarengo et al., 1991) eta elikagaien urritasunarekin (Benito et al., 2017) lotu da. Schmorl metodoa (Pearse, 1985) erabili zen lipofuszinak detektatzeko. Ebaketak (8 µm) CM3050S Leica kriotomo batean moztu ziren

-25 °C-ko ganbera tenperaturan, beirazko prestakin epeletan bildu eta -40 °C-tan gorde ziren tindatzeko behar izan arte. Gertakinak (talde bakoitzeko 10 liseri-guruin) %2,5 NaCl zuen Baker-en kaltzio formolean fixatu ziren 15 minutuz 4 °C-tan. Ebaketak ur destilatutan garbitu ondoren, % 1 kloruro ferrikoa eta % 1 potasio ferrizanuroa 3:1 proportzioan zituen erreakzio medioan murgildu ziren 5 minutuz. Ondoren, ebaketak % 1-eko azido azetikotan garbitu ziren minutu batez. Azkenik, ebaketak ur destilatutan garbitu eta Kaiser-en glizerina gelatinarekin muntatu ziren. Vv_{LPF} zitosolaren bolumen-dentsitateariko kuantifikatu zen BMS softwarea erabiliz liseri-zelulen lisosomen egitura kuantifikatzeko, lehenago azaldu bezala, x40 handitze objektiboa erabiliz. Vv_{LPF} lipofuszinen bolumenaren eta zitosolaren bolumenaren arteko erlazioa ($Vv_{LPF} = V_{LPF}/V_C$) kalkulatu zen, non V_{LPF} = lipofuszinen bolumena eta V_C = zitosolaren bolumena.

Liseri-guruinean lipido neutroen (NL) metaketa xenobiotiko organikoen esposizioarekin, estres ez espezifikoarekin eta elikadura egoerarekin erlazionatu da (Cancio et al., 1999, Marigómez eta Baybay-Villacorta, 2003, Shaw et al., 2011). NL edukiaren zehaztapena liseri-guruinetako 8 µm-ko lodierako ebaketa izoztuetan egin zen (n=10), kriostatoan lortutako -25°C-ko ganberako tenperaturan. Prestakinak -40 °C-tan gorde ziren eta Lillie eta Ashburn-en Oil Red O (ORO) metodoa jarraituz tindatu ziren (Culling, 1974). NL edukia ehun konektiboarekiko BMS softwarea erabiliz neurtu zen (lehenago deskribatuta), x40 handitze objektiboa erabiliz. Vv_{NL} lipido neutroen bolumenaren eta ehun konektiboaren bolumenaren arteko erlazio gisa kalkulatu da $Vv_{NL} = V_{NL}/V_{CT}$, non V_{NL} = lipido neutroen bolumena eta V_{CT} = ehun konektiboaren bolumena (Marigomez eta Baybay-Villacorta, 2003).

Ehun mailako biomarkatzaileak eta histopatología

Parafina ebaketak (5 µm) hematoxilina-eosinaz (H/E) tindatu ziren, liseri-guruinaren, gonadaren eta zakatzen osotasuna aztertzeko.

Vv_{BAS} estres orokorreko biomarkatzaile bat da, moluskuen liseri-albeoloetan estres baldintzetan ematen den zelula basofiloen hazkunde erlatiboa neurtzen duena (Zorita et al., 2006, Rementeria et al., 2016, Benito et al., 2017).

CTD ratio altu batek moluskuen liseri-guruinaren osotasunaren galera adierazten du, eta estres kimikoak edo elikadura egoera eskasak eragin dezake (Mújica et al., 2015b, Benito et al., 2017).

Biak, Vv_{BAS} eta CTD, estereologiaren bidez kuantifikatu ziren (n=10) zelula-mota osaeran, liseri-epitelioaren batez besteko lodieran eta ehun konektibo kopuruan aldaketak gertatu diren edo ez adierazteko. Ausaz aukeratutako hiru eremutan zenbaketak egin ziren liseri-guruinen prestakin batean muskuilu bakoitzeko (10 muskuilu talde bakoitzeko). Gertakinak 40× objektiboan behatu ziren (handitzea totala ~ 400×) argi-mikroskopio bati loturiko marrazketa-hodi bat erabiliz. M-168 Weibel gratikula erabilera anitzeko sistemaren (Weibel, 1979) bertsio sinplifikatu bat erabili zen, eta zelula basofiloak (b), liseri-zelulak (d), lumen dibertikularrak (l) eta ehun konektibo interstiziala (c) erregistratu ziren. Vv_{BAS} Delesseren printzipioaren arabera kalkulatu zen (Weibel, 1979), $Vv_{BAS}=V_{BAS}/V_{EP}$ gisa, non V_{BAS} zelula basofiloen bolumena den eta V_{EP} liseri-guruinen epitelioaren bolumena den. CTD ratioa CTD = c/(b + d + l) gisa kalkulatu zen.

Atrofia indize gisa neurtutako muskuiluen liseri-albeoloen mehetze epiteliala estres orokorraren adierazgarri izan daiteke (Kim et al., 2006, Garmendia et al., 2011). Liseri-albeoloen atrofia indizearen larritasuna 0tik 4rako (n=20) zenbakizko kalifikazioa erabiliz baloratu zen Kim et al. (2006)-ek deskribatutako moduan:

- 0: lumen ia ikusezina duten dibertikulu normalak.
- 1: epitelioaren lodiera normalaren erdia baino handiagoa duten hodiak. Hodi normalak eta partzialki atrofiatuak batera agertzen dira.
- 2: liseri-epitelioaren lodiera normalaren erdia.
- 3: hodi nabarmen atrofiatua, liseri-epitelioaren lodiera normalaren erdia baino gutxiagorekin.
- 4: liseri-epitelio oso meheak, ia hodi guztiak kaltetuta.

Analisi histopatologikoei dagokienez, laginketa kanpaina bakoitzeko 20 muskuiluren gertakinak banan-banan aztertu ziren argi-mikroskopioan 10×, 20× edo 40× objektiboekin. Puntuazio kuantitatiboak egin ziren (emaitzak ez dira erakusten) intzidentziaren zenbaketa mantenduz, gertakina eskaneatu ahala, gertakari bakoitza

behin baino gehiagotan aztertzea ekiditeko. Parasito digeneoen, MPX zelula barneko ziliatuen eta *Rickettsia* bezalako organismoen prebalentzia aztertu zen. Horrez gain, zenbait ehun egoeraren prebalentzia aztertu zen, infiltrazio hemozitikoaren kasuak barne, fokatua eta difusoa bereizi gabe, granulozitomak eta zelula arreen (gorputz zeroideak edo pigmentu-zelulak) agregatuak eta atresia folikularra ere aztertu ziren.

Biomarkatzaileen erantzun integratuaren indizea (IBR/n)

IBR indizea bost erantzun biologikoren integrazioan oinarritu zen, biokimikotik ehun mailara (CAT, LPO, Vv_{NL}, Vv_{BAS} eta CTD), erantzunak antolakuntza biologiko ezberdinetan irudikatzeko, Beliaeff eta Burgeot (2002), Broeg eta Lehtonen (2006) eta Marigómez et al. (2013)-en arabera. Kalkuluak aldagai anitzeko metodo grafiko batean oinarritu ziren, prozedura honen arabera: (1) lagin bakoitzaren batez bestekoa eta desbideratze estandarraren kalkulua; (2) lagin bakoitzaren datuen estandarizazioa: $x_i' = (x_i-x)/s$; non, x_i' = biomarkatzailearen balio estandarizatua; x_i = lagin bakoitzeko biomarkatzaile baten batez besteko balioa; x = x_i-ren batez besteko balio orokorra, alderatutako lagin guztietatik kalkulatua; s = x_i-ren desbideratze estandarra, lagin guztietatik kalkulatua; (3) lagin bakoitzeko lortutako balio estandarizatua datu multzoko gutxieneko balio estandarizatu absolutuari gehitzea: $y_i = x_i' + |x_{min}'|$; (4) radar grafikoaren eremu triangeluarrak kalkulatzea $A_i = (0,59 \times (y_i \times y_i+1)/2$, non ''$y_i$'' eta ''$y_i$+1'' biomarkatzaile bakoitzaren eta hurrengoaren balio estandarizatuak baitziren. Izar grafikoan 0,59 sin α da (α: angelu erradiala izar grafiko pentagonal baterako; $\alpha=2\pi/5$); eta (5) IBR indizearen kalkulua Irudikatutako eremu triangeluarren guztien batuketa dena ($IBR=\Sigma A_i$) (Beliaeff eta Burgeot, 2002). IBR balioa datu multzoko biomarkatzaile kopuruaren menpe zuzenean dagoenez, lortutako IBR balioa IBR/n kalkulatzeko erabilitako biomarkatzaile kopuruarekin zatitu zen (Broeg eta Lehtonen, 2006).

Analisi estatistikoak

Analisi estatistikoa SPSS/PC+ V.24 pakete estatistikoaren laguntzaz egin zen. (SPSS Inc., Microsoft Co.). Normaltasun frogak gainditu zituzten datu kuantitatiboetarako, norabide bakarreko ANOVA eta ondorengo Duncan-en post-hoc

proba aplikatu ziren batez besteko balioen bikoteen arteko konparazio anitzak egiteko ($p < 0,05$). Lortutako emaitza erdikuantitatiboetarako eta normaltasun frogak gainditu ez zituzten emaitza kuantitatiboetarako, Kruskal Wallis-en froba ez parametrikoak egin ziren laginketa guneen eta urtaroen arteko bariantzak alderatuz ($p<0,05$). Ehun bigunen eduki kimikoa, alterazio histopatologikoen prebalentzia eta IBR/n balioak laginketa gune eta urtaroen artean alderatzeko, Z-score erabili zen ($p<0,05$). Sateliteen datuak R sistema erabiliz prozesatu ziren (R Core Team, 2018).

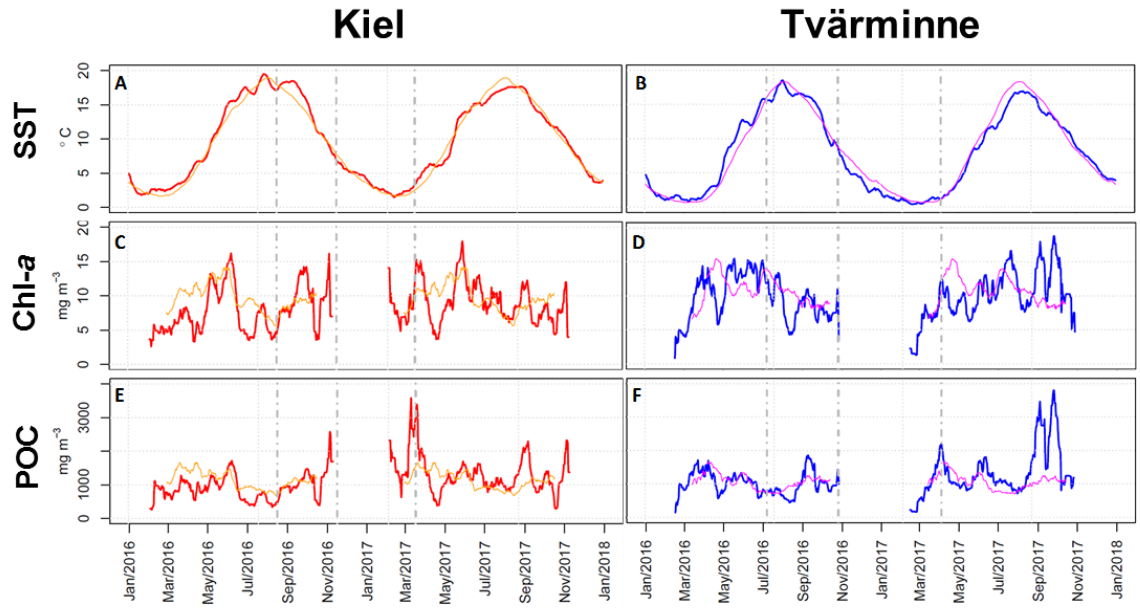
EMAITZAK

Ingurugiro faktoreen urrutiko detekzioa

SST neurketek Kielen (2A. irud.) eta Tvärminnen (2B. irudia) udan tenperaturak antzekoak zirela erakutsi zuten. 2016ko udako laginketa baino hilabete lehenago, tenperaturak egonkorrak izan ziren Kielen. Tvärminnen SST oraindik bilketa aurreko hilabetean igotzen ari zen. Kielen udazkeneko laginketa Tvärminneko udazkeneko laginketa baino tenperatura baxuagoak zirenean egin zen. 2017ko udaberrian Kiel-en SST apur bat handiagoa izan zen eta jada handitu egin zen muskuiluak bildu zirenean, baina Tvärminnen oraindik ez zen nabarmen igotzen ari laginketa garaian.

Udan, muskuilu bilketaren aurreko egunetan chl-a kontzentrazioa baxua zen Kielen (serie historikoan baino zertxobait baxuagoa) (2C. irudia), Tvärminnen, berriz, udako laginketak chl-a kontzentrazio altuko aldi jarraitu baten ondoren egin ziren (altuak sasoiko balio tipikoekin alderatuta) (2D. irudia). Kielen, chl-a kontzentrazioen sasoiko gailurra udazkenean erregistratu zen, eta Tvärminnen, berriz, udazkeneko laginketa baino lehen. Udaberriko laginketan, Kielen chl-a-ren gorakada izugarria erregistratu zen, aldi honetako balioak 17 urteko errekorretik eratorritako urtaro ziklo leundukoak baino handiagoak izan ziren. Tvärminne-n, udaberriko laginketak urteko lehen chl-a gailurrean egin ziren.

Kielen, udan POC kontzentrazioa urte anitzeko urtaro zikloaren arabera normalean erregistratutakoa baino txikiagoa izan zen (2E. irudia). Tvärminne-n urteko garai berean erregistratu ohi zirenen antzekoak ziren (2F. irudia). Kielen, POC mailen igoera gertatu zen udazkenean, eta Tvärminnen, udazkeneko laginketa baino lehen igoera txiki bat ikusi ahal izan zen. Kielen POC mailarik altuenak udaberriko laginketatik gertu ikusi ziren eta urtaro leunduko zikloaren balioak baino askoz ere altuagoak izan ziren. Tvärminne-n, udaberrian muskuilu bilketaren garaian POC kontzentrazio gailur bat antzeman zen, urte gehienetan ikus daitekeen gailurra baino apur bat lehenago.



2. Irudia Bi urteetako urrutitik detektatutako datuen grafikoak. Itsaso gainazaleko tenperatura (SST) (Kiel A, Tvärminne B), a-klorofila kontzentrazioa (Chl-a) (Kiel C, Tvärminne D) eta karbono organiko partikulatua (POC) (Kiel E, Tvärminne F). marra laranjak (A, C, E) eta moreak (B, D, F) azken 17 urteetako eguneko batez besteko urtaroko ziklo leunduak irudikatzen dituzte, eta lerro bertikal ez jarraiek laginketak noiz egin ziren.

Ehunetako PAH edukia

PAH bakarren ehunen edukia gehienetan konposatuen detekzio mugetatik behera edo oso gertu zegoen (1. taula). Kontzentrazio handienak aurkezten zituzten PAH-ak fluorantenoa eta pirenoa izan ziren, nahiz eta baliorik altuenak ez ziren estatistikoki desberdinak izan kontzentrazio baxuagoak erakusten zituzten laginekin alderatuta. Neurtutako 15 PAH-en batura nabarmen handiagoa izan zen Kielen udazkenean bildutako muskuiluetan gainerako laginekin alderatuta.

1. Taula: *Mytilus trossulus*-en ehunetan aurkitutako PAH kontzentrazioak (µg/kg pisu lehorra). Asteriskoek desberdintasun esangarriak adierazten dituzte ($p < 0.05$). DM, detekzio muga. -, detekzio mugaren azpitik

	DM	Kiel Uda	Kiel Udazkena	Kiel Udaberria	Tvärminne Uda	Tvärminne Udazkena	Tvärminne Udaberria
Anthracene	9	-	-	-	-	-	-
Acenaphtene	9	-	-	-	-	-	-
Acenaphtylene	9	-	-	-	-	-	-
Benz(a)anthracene	18	-	18.9	-	-	-	-
Benzo(a)pyrene	18	-		-	-	-	-
Benzo(b)fluoranthene	18	-	22.5	-	-	-	-
Benzo(ghi)perylene	18	-	-	-	-	-	-
Benzo(k)fluoranthene	18	-	-	-	-	-	-
Dibenz(a,h)anthracene	18	-	-	-	-	-	-
Phenantrene	9	-	18	11.7		-	-
Fluoranthene	9	17.1	63.9	36	9.9	-	14.4
Fluorene	9	-	-	-	-	-	-
Indeno(1,2,3-cd) pyrene	18	-	-	-	-	-	-
Chrysene	18	-	-	-	-	-	-
Pyrene	18	15.3	48.6	22.5	-	-	12.6
∑PAHs		32.4	171.9*	70.2	9.9	-	45

Ehunetako aztarna metalen edukia

Ehunen aztarna metalen kontzentrazio batzuek desberdintasun nabarmenak erakutsi zituzten taldeen artean (2. taula). Udan bildutako Kiel-eko muskuiluak Hg, Zn eta Mn kontzentrazio nabarmen handiagoak zituzten gainerako taldeekin alderatuta.

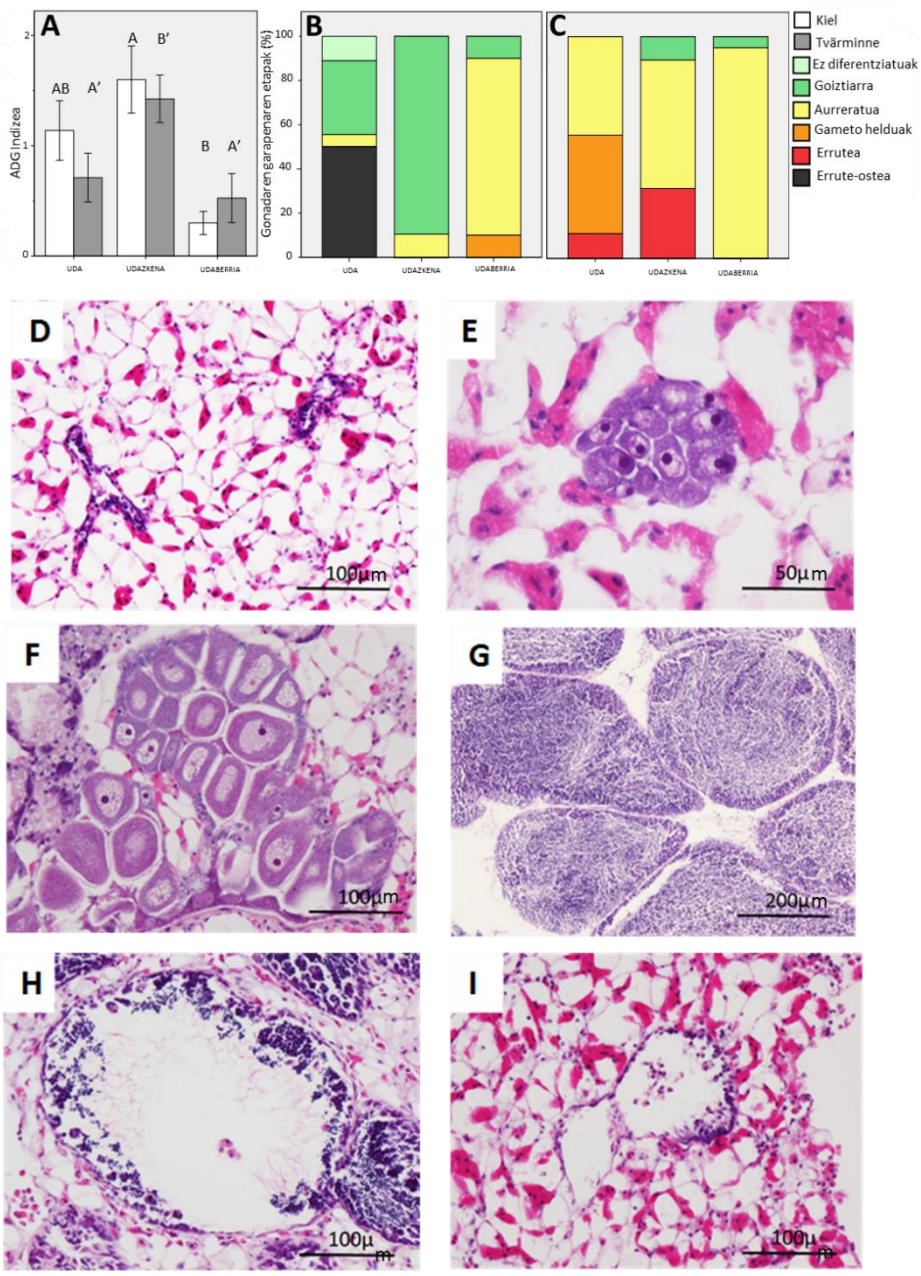
2. Taula: *Mytilus trossulus*-en ehunetako metalen kontzentrazioa (µg/g pisu lehorra). Asteriskoek desberdintasun esangarriak adierazten dituzte ($p < 0.05$).

	Kiel Uda	Kiel Udazkena	Kiel Udaberria	Tvärminne Uda	Tvärminne Udazkena	Tvärminne Udaberria
Li	0.36	0.28	0.37	0.27	0.22	0.09*
Al	44.63	52.58	37.67	113.10*	81.36	30.18
Sr	23.69	18.35	26.35	50.54*	37.98	26.27
Mo	0.46	0.48	0.47	0.58	0.60	0.39
Ag	0.03	0.02	0.02	0.05	0.06	0.04
Sn	0.17	0.22	0.11	0.08	0.037	0.01
Sb	0.02	0.03	0.02	0.02	0.01	0.01
Ba	10.75	6.39	2.07	8.87	5.86	3.35
W	0.03	0.03	0.02	0.04*	0.09	0.02
Hg	0.31*	0.19	0.22	0.20	0.16	0.11
Tl	0.01	0.01	0.01	0.01	0.01	0.01
Pb	1.53	0.98	1.11	0.70	0.61	0.41
Ti	10.96	10.52	13.77	19.78	17.64	10.86
Co	0.43	0.26	0.25	0.49	0.41	0.27
Cu	8.25	6.43	6.66	8.08	6.94	6.67
Zn	121.40*	92.22	96.28	76.82	89.17	69.64
As	5.50	5.49	7.47	9.60	9.37	6.08
Cd	0.63	0.48	0.47	1.21	1.00	0.59
V	0.51	0.83	0.58	0.48	0.53	0.18
Cr	0.45	0.41	0.41	0.91	0.78	0.40
Mn	114.52*	27.54	25.33	53.34	27.98	22.88
Fe	211.08	219.52	160.75	312.49	288.30	155.43
Ni	2.13	1.32	0.92	2.39	2.14	1.31
Se	2.29	2.11	2.17	2.11	2.02	1.85

Zelula adipogranularren indizea

Zelula adipogranularren indizeak ez zuen desberdintasun esangarririk erakutsi laginketa tokien artean, baina baliorik altuena udazkenean neurtu zen eta baxuena udaberrian (3A. irudia).

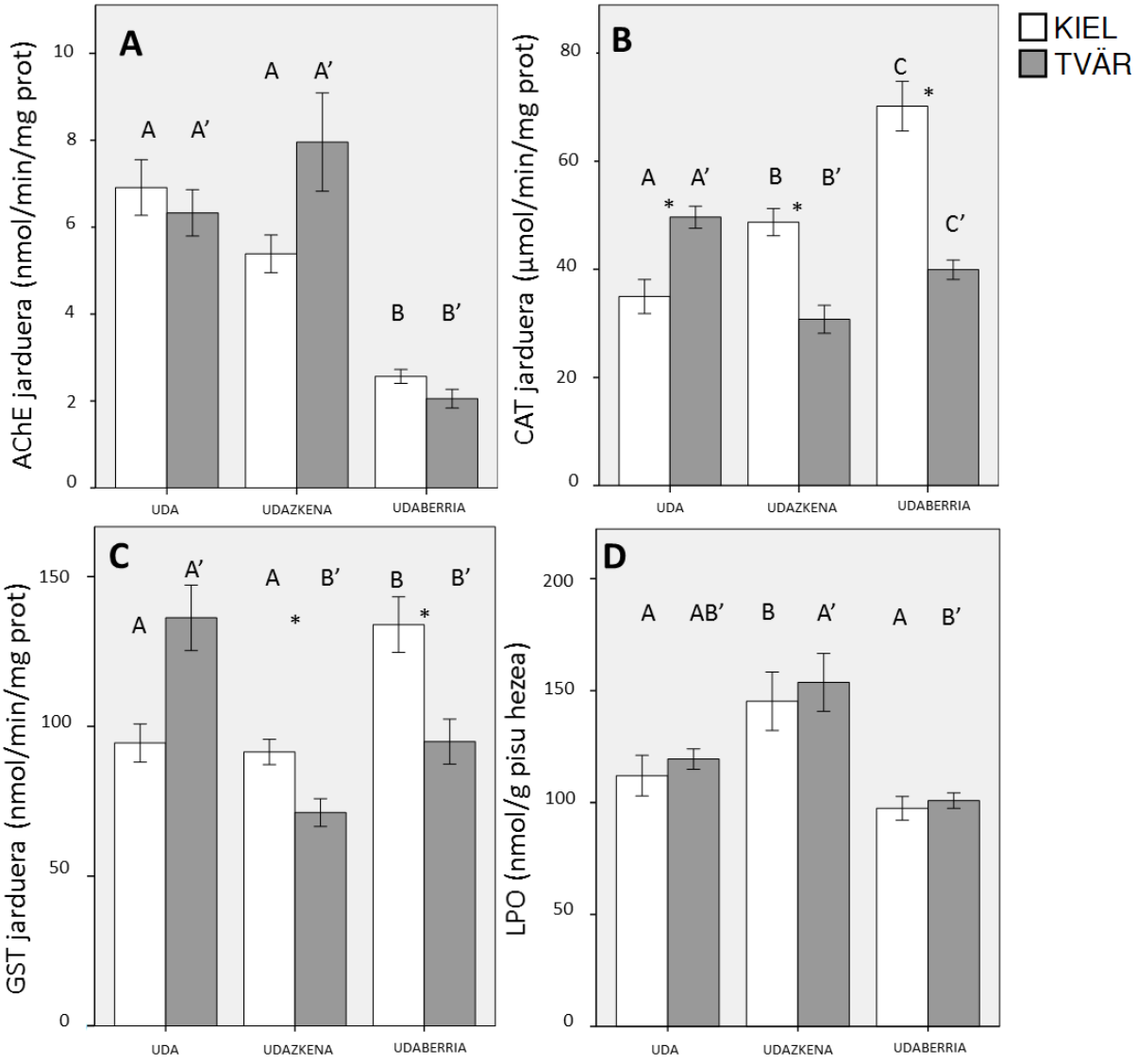
Gametoen garapen etapetan desberdintasunak agertu ziren urtaro eta laginketa guneari dagokienez. Kiel-en udan, muskuiluen erdia errute ondorengo fasean zegoen (3I. irudia), eta aztertutako muskuiluen % 33-k gametogenesi goiztiarra erakusten zuen, % 11-k gameto ez desberdinduak (3D. irudia) eta % 5 fase gametogeniko aurreratuan zeuden. Udazkenean bildutako muskuilu geihenak egoera gametogeniko goiztiar batean zeuden (% 90), eta, neurri txikiagoan, gametogenesi aurreratuan (% 10). Udaberrian muskuilu gehienak gametogenesi egoera aurreratuan zeuden (% 80) (3B, 3F irudiak), eta batzuk gametogenesiaren hasierako fasea (% 10) (3E. irudia) edo gameto helduak (% 10) erakusten zituzten (3G. irudia). Tvärminne-n (3C. irudia) udan, muskuiluen % 44 egoera gametogeniko aurreratuan zegoen eta beste % 44k gameto helduak erakusten zituen eta azken % 11 errute fasean zegoen (3H. irudia). Udazkenean muskuiluen zatirik handiena (% 58) fase gametogeniko aurreratuan zegoen, % 31k errute fasea erakusten zuen eta % 10 gametogenesi goiztiarrean zeuden. Udaberrian muskuilu gehienak egoera gametogeniko aurreratuan zeuden (% 95), eta gainerakoek gametogenesi goiztiarrean (% 5).



3. Irudia: *Mytilus trossulus*-en mantuan zelula adipogranularren indizea erakusten duen grafikoa (n=20) (A), barrek errore estandarra eta letrek urtaroen arteko desberdintasun estatistikoak irudikatzen dituzte (p<0.05). B-k Kiel-eko muskuiluen gonadaren garapenaren etapen portzentaiak erakusten ditu eta C-k Tvärminne-koak (n=20). Gonada ebaketa desberdinen detaileak (haematoxylina-eosinaz tindatuak). D, gameto ez diferentziatuak; E, gametogenesi goiztiarra (emea); F, gametogenesi aurreratua (emea); G, gameto helduak (arra); H, errute fasea eta I, errute osteko fasea.

Muskuiluen zakatzetan neurtutako AChE jarduerak ez zuen desberdintasun estatistikorik erakutsi bi tokien artean (4A. irudia). Hala ere, udaberrian lortutako balioak udan eta udazkenean neurtutakoak baino nabarmen baxuagoak izan ziren bi lekuetan. Liseri-guruinean CAT jarduerak alde nabarmenak erakutsi zituen tokien eta urtaroen artean (4B. irudia). Kiel-eko muskuiluek udaberrian izan zuten jarduerarik handiena eta udan baxuena. Udazkenean CAT jarduerak gora egin zuen berriro, tarteko balioetara iritsiz. Tvärminne-n, CAT batez besteko jarduerarik handiena udan neurtu zen eta baxuena udazkenean, tarteko balioak udaberriko laginetan erregistratu zirelarik. Kiel-eko muskuiluen liseri-guruinean GST jarduera altuena udaberrian izan zen, desbertinasuna estatistikoki esanguratsua izanik udako eta udazkeneko balioekin konparatzerakoan eta Tvärminne-n udaberrian bildutako muskuiluetan neurtutakoekin alderatuta (4C. irudia). Tvärminne-ko muskuiluetan GST jarduerarik handiena udan neurtu zen, estatistikoki esanguratsua izanik desberdintasuna udaberriko eta udazkeneko mailekin konparatzerakoan eta Kielen udan bildutako muskuiluekin alderatuta.

LPO mailak nabarmen handiagoak ziren bi lekuetan udazkenean, Tvärminne-n udan lagindutako muskuiluek tarteko balioak erakusten zituzten bitartean (4D. irudia). Lekuen artean ez zen desberdintasun esanguratsurik aurkitu LPO mailen artean.

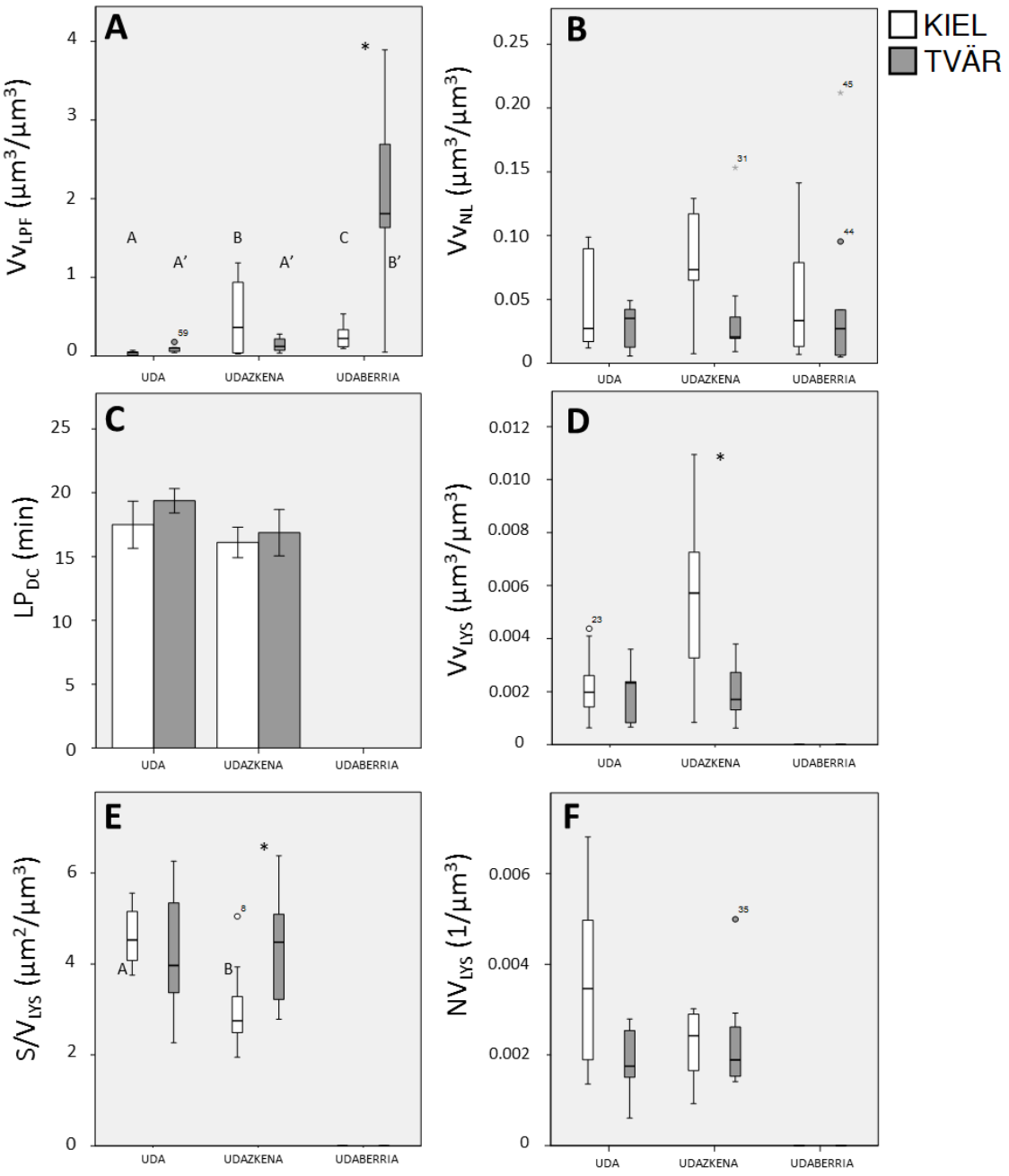


4. Irudia: A, Azetilkolinesterasa jarduera zakatzetan (n = 30) eta B, katalasa jarduera (n = 15); C, Glutation S-transferasa jarduera (n = 15) eta D, lipidoen peroxidazio mailak (n = 15) liseri-guruinean. Barrek errore estandarra irudikatzen dute; letrek urtaroen arteko desberdintasun estatistikoak irudikatzen dituzte eta asteriskoek urtaro berdinean lekuen arteko desberdintasun estatistikoak ($p < 0.05$).

Zelula-mailako biomarkatzaileak

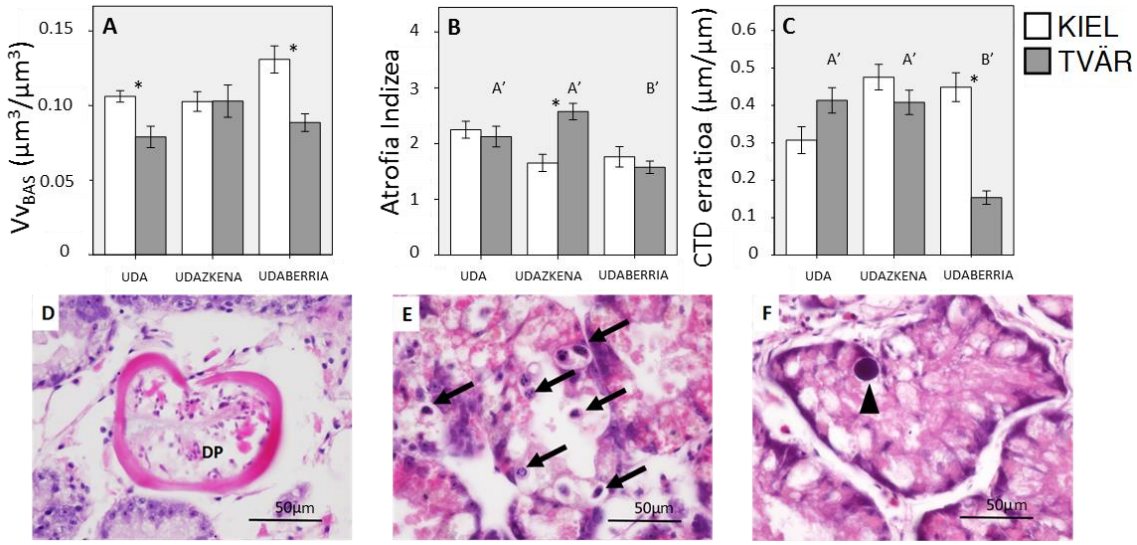
Muskuiluen liseri-guruinean lipofuszina edukiak desberdintasun nabarmenak erakutsi zituen hiru urtaroen artean bi laginketa guneetan (5A. irudia). Kiel-en kasuan udazkenean bildutako muskuiluetan neurtu ziren baliorik altuenak, udaberrian bitarteko balioak neurtu ziren eta udan baxuenak. Tvärminne-n udaberrian ikusi zen lipofuszina eduki esanguratsuena. Leku honetan ez zen udan eta udazkenean bildutako muskuiluen

arteko alderik antzeman. Lipido neutroen edukiari buruzko desberdintasun estatistikorik ez zen antzeman laginketa lekuen eta urtaroen artean (5B. irudia). Udaberriko laginetako lisosomen neurketak ezin izan dira fidagarritasunez egin tindaketa kalitate eskasa eta lipofuszina itxurako granuluak sortutako interferentziak direla eta. Egonkortasun lisosomiko probak ez zuen alde handirik erakutsi urtaroen edo tokien artean (5C. irudia). Bolumen dentsitate lisosomikoak ez zuen Kiel-en edo Tvärminne-n desberdintasun handirik erakutsi udako eta udazkeneko laginen artean, nahiz eta balioak nabarmen handiagoak izan udazkenean Kiel-en Tvärminne-rekin alderatuta (5D. irudia). Azalera/bolumen erlazio lisosomikoak udatik udazkenera jaitsiera nabarmena izan zuen Kiel-eko muskuiluetan, eta Tvärminne-ko laginetan ez zen urtaroen arteko alde nabarmenik ikusi (5E. irudia). Kiel-en ratioa nabarmen handiagoa izan zen udazkenean Tvärminne-ko muskuiluekin alderatuta. Ez zen desberdintasun esanguratsurik aurkitu urtaroen eta tokien arteko zenbaki dentsitate lisosomikoan (5F. irudia).



5. Irudia: : A, Liseri-zeluletan metatutako lipofuszinen kontzentrazioa B, liseri-zeluletan metatutako lipido neutroen metaketa. C, egonkortasun lisosomikoaren testaren batez bestekoak eta hurrengo emaitzak erakusten dituzten kutxa diagramak: D, bolumen dentsitate lisosomikoa; E, azalera/bolumen erlazio lisosomikoa eta F, dentsitate numeriko lisosomikoa (n = 10). C grafikoan barrek errore estandarra irudikatzen dute, letrek urtaroen arteko desberdintasun estatistikoak eta asteriskoek urtaro berean lekuen artekoak ($p < 0.05$).

Zelula basofiloen bolumen dentsitateak ez zuen urtaro arteko aldakortasunik erakutsi (6A. irudia). Hala ere, udaberriko eta udako laginetan Kiel-eko muskuiluetan neurtutako balioak Tvärminne-koetan baino nabarmen handiagoak ziren. Atrofia indizeak ez zuen sasoiko eredurik erakutsi Kiel-en, eta Tvärminne-n, berriz, udaberrian bildutako muskuiluek atrofia maila nabarmen txikiagoa zuten udako eta udazkeneko laginekin alderatuta (6B. irudia). Tokien arteko desberdintasun estatistiko esanguratsu bakarrak udazkenean aurkitu ziren Tvärminne-ko muskuiluek Kiel-ekin alderatuta atrofia maila handiagoa zutelarik. Ehun konektibo eta liseri-ehunen arteko erlazioa (CTD) ez zen nabarmen aldatu Kiel-en urtaroen artean (6C. irudia), baina Tvärminne-n muskuiluek CTD nabarmen txikiagoa izan zuten udaberrian uda eta udazkenarekin alderatuta. Udaberrian CTD ratioa nabarmen handiagoa izan zen Kiel-en Tvärminne-rekin alderatuta.



6. Irudia: A, zelula basofilikoen bolumen dentsitatea (n =10); B, atrofia indizea (n =20) eta C, ehun konektibo eta liseri-ehunen arteko erratioa (n =10). Barrek errore estandarra irudikatzen dute, letra desberdinek urtaroen arteko desberdintasun estatistikoak irudikatzen dituzte eta asteriskoek urtaro berdinean lekuen arteko desberdintasun estatistikoak irudikatzen dituzte ($p<0.05$) eta mikrografiak (haematoxylina-eosinaz tindatuta) *Mytilus trossulus* liseri-ehunak erakusten: D, parasito digeneo bat; E, MPX zelula barneko ziliatua and F, *Rickettsia* itxurako organismoa. DP, parasito digeneoa; geziak, MPX ziliatuak eta triangeluak Rickettsia itxurako organismoa.

Aurkitutako patologiak hanturazko erantzunekin (zelula hemozitiko eta arreen infiltrazioekin), granulozitomekin eta obozitoen atresiarekin erlazionatuta zeuden. Aurkitutako parasitoak digeneoak eta MPX zelula barneko ziliatuak ziren (3. taula). Tvärminne-ko muskuiluetan infiltrazio hemozitikoak aurkitu ziren udaberrian (banakoen % 85), udan (% 70) eta udazkenean (% 80). Kiel-en infiltrazio hemozitikoen prebalentzia handiena udan detektatu zen (% 55). Laginketa tokien eta urtaroen artean ez zen desberdintasun estatistiko esanguratsurik ikusi. Liseri-ehunean zelula arreen infiltrazioen prebalentzia nabarmen handiagoa izan zen udaberrian Tvärminne-ko muskuiluetan (% 70), prebalentzia baxua izan zelarik beste lagin guztietan (% 0-25). Gonada ehunetan zelula marroien infiltrazioaren prebalentzia nabarmen handiagoa izan zen Tvärminne-ko muskuiluetan (% 80) udan, beste bi urtaroetan prebalentzia txikiagoak izan ziren (udaberria % 45, udazkena % 0). Kiel-en prebalentzia oso baxuak izan ziren orokorrean (udaberria % 0, uda % 10 eta udazkena % 5).

Granulozitomak Tvärminne-n udaberriko laginetan bakarrik ikusi ziren (% 20). Kielen obozitoen atresiaren prebalentzia handiena udaberrian izan zen (% 90), eta jaitsi egin zen udan (% 13) eta udazkenean (% 7). Tvärminne-ko muskuiluak udaberrian (% 100) izan zuen prebalentziarik handiena, eta udan (% 87) eta udazkenean (% 61) balioak baxuagoak izan ziren arren, aldeak ez ziren estatistikoki esanguratsuak tokiak eta urtaroak alderatuz gero.

Parasitoen agerpena orokorrean baxua izan zen bi azterketa guneetan. Parasito digeneoak zertxobait ohikoagoak ziren Kiel-en (udaberrian % 10, udan % 14 eta udazkenean % 5) Tvärminne-n baino (udaberrian % 5, udan eta udazkenean % 0) (6D. irudia). MPX ziliatuen infekzio intentsitateak ehun ebaketa bakoitzeko 50 indibiduotik beherakoak izan ziren beti (6E. irudia). MPX prebalentzia baxua zen Kiel-en (udaberrian eta udan % 5, udazkenean % 10), Tvärminne-n, berriz, udaberrian baino ez ziren aurkitu prebalentzia nabarmen handiagoarekin (% 26). Liseri-guruinean *Rickettsia* antzeko organismoak (RLO) ez ziren aurkitu Kiel-en, Tvärminne-n haien prebalentzia oso baxua zen (udaberrian % 5, udan % 10en eta udazkenean % 5en) eta urtaroen aldeak ez ziren esanguratsuak izan (6F. irudia). Ebaketa bakarrean RLO kopururik handiena 14 izan zen.

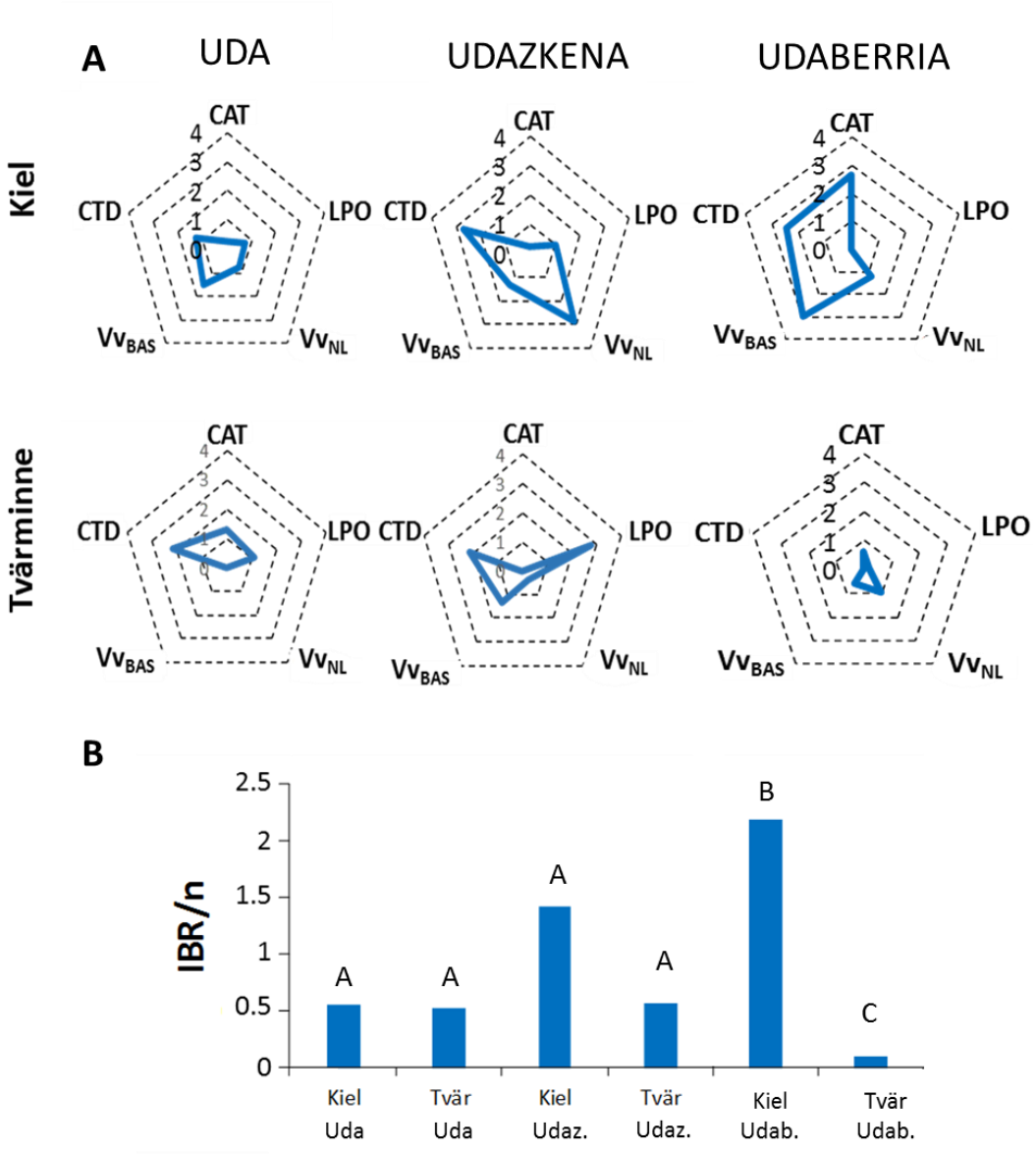
3. Taula: Hurrengo patologia eta parasitoen prebalentziak (%): infiltrazio hemozitikoa, liseri-guruinean eta gonadan aurkitutako zelula arreen infiltrazioak, granulozitomak, obozitoen atresia, parasito digeneoak, MPX ziliatuak eta *Rickettsia* antzeko organismoak liseri-guruinean (n=20). Asteriskoak taldeen arteko desberdintasun estatistikoak irudikatzen ditu (p<0.05). LG: Liseri-guruina, GO: Gonada.

	Kiel Uda	Kiel Udazkena	Kiel Udaberria	Tvärminne Uda	Tvärminne Udazkena	Tvärminne Udaberria
Inf. hemozitikoa	55	25	10	70	80	85
Zelula arreak (LG)	25	15	5	0	0	70*
Zelula arreak (GO)	10	5	0	80*	0	45
Granulozitomak	0	0	0	0	0	20*
Atresia	13.33	6.67	90	87.5	61.54	100
Par. Digeneoak	14.29	5	10	0	0	5
MPX	5	10	5	0	0	26.32*
RLO (LG)	0	0	0	10	5	5

Biomarkatzaileen erantzun integratuaren indizea (IBR/n)

IBR-rako hautatutako biomarkatzaileak honako hauek izan dira: CAT jarduera (estres oxidatiboaren defentsak) eta LPO mailak (kalte oxidatiboa) liseri-guruinean maila biokimikoan; Vv_{NL} liseri-zeluletan maila azpi-zelularrean; Vv_{BAS} maila zelularrean eta CTD ratioa ehun mailako biomarkatzaile gisa. Biomarkatzaile hauek antolakuntza maila eta funtzio biologiko desberdinak adierazten dituztelako aukeratu dira eta ikerketa honetan neurtutako erantzun biologikoen ikuspegi holistiko laburtua lortzeko aukera ematen dutelako. Erantzun biologikoko bost parametroek irudikatutako sei profilek urtaroko eta denborazko aldakortasuna erakusten zuten izar edo radar grafikoetan (7. irudia). CAT, Vv_{BAS} eta CTD-ek udaberrian lagindutako Kiel-eko muskuiluetan izan zuten erantzun handiena, eta IBR/n baliorik altuena lortu zuten, estatistikoki besteekiko desberdina izanik. Orokorrean, hautatutako biomarkatzaileetan erantzun maila baxuenak Tvärminne-n neurtu ziren udaberrian, beraz, IBR/n balioa baxuena da. Bi lekuetan udan lagintzen diren muskuiluek, orokorrean, erantzun maila ertaina erakusten dute. Kiel-en

udazkenean bildutako muskuiluetan Vv_{NL} erantzuna handiagoa da, CTD erantzuna udaberriko muskuiluetan bezain handia da eta IBR/n balioa Kiel-en udako eta Tvärminne-ko udazkeneko balioak baino handiagoa da, nahiz eta aldeak estatistikoki esanguratsuak ez izan. Udazkenean lagindutako Tvärminne-ko muskuiluek LPO erantzun handiena izan zuten, gainerako biomarkatzaileek tarteko erantzunak erakusten dituzte eta IBR/n balioa udan Tvärminne-n bildutako muskuiluen antzekoa da.



7. Irudia: A, Biomarkatzaileen izar grafikoak eta B, IBR/n indizea Kiel udaberrian, udan eta udazkenean eta Tvärminne udaberrian, udan eta udazkenean. Letrek taldeen arteko desberdintasun estatistikoak irudikatzen dituzte ($p<0.05$). CAT, katalasa jarduera; LPO, lipidoen peroxidazioa; Vv_{NL}, liseri-guruinean metatutako lipido neutroen kontzentrazioa; Vv_{BAS}, zelula basofiloen bolumen dentsitatea; CTD, ehun konektibo eta liseri-ehunen arteko proportzioa.

EZTABAIDA

Itsaso Baltikoko ingurugiro faktoreak (tenperatura eta gazitasunak, besteak beste) oso aldakorrak dira denbora eta espazioan, biomarkatzaileen erantzunetan eragina izan dezakeena. Horregatik, aldagai fisiko kimiko eta ekologiko desberdinak teledetekzioaren bidez neurtzeak (Frouin et al., 1989, Stramski et al., 2008, Hu et al., 2012, Kilpatrick et al., 2015) abantaila logistiko bat eman dezake informazioa biltzeko aldagai ekologikoen laginketa edota neurketa osagarrien beharrik gabe. Hala ere, kontuan hartu behar da sateliteen datuek aztertutako aldagaien eskualde ezaugarriak soilik irudika ditzaketela eta erradiazio atmosferikoaren transmisiok sortutako arazoen eraginpean egongo direla (hodei estalduraren ondorioz falta diren datuak kasu). Hori dela eta, *in situ* datuek sateliteen datuek baino eskalen parekatze hobea duten neurketak eskaintzen dituzte, batez ere kostaldetik oso gertu dauden eskualdeetan.

Itsasoaren gainazaleko tenperatura parametro kritikoa da Itsaso Baltikoko muskuiluentzat, *upwelling* prozesuetan eragiten baitu eta, ondorioz, ur zutabearen nahasketa bertikalarekin eta fitoplanktonaren dinamika zehazten duten mantenugaien mobilizazioarekin lotuta baitago (Wasmund et al., 2008). Lan honetan, ez da anomaliarik aurkitu SST-ri dagokionez, laginketa urteak urtaro zikloarekin alderatuz gero, nahiz eta tenperaturaren joerak desberdinak diren bi tokietan. Tvärminne-n tenperatura baxuagoko aldi luzeagoak daude Kiel-ekin alderatuz gero, eta udako tenperatura altuen aldia laburragoa da. Guneak alderatzean sasoiko joerak hartu behar dira kontuan, data jakin batean lagintzen diren muskuiluak ingurumen baldintza ezberdinen eraginpean egon daitezkeelako.

SST-ren antzeko joerak ikusi daitezke Chl-a eta POC kontzentrazioei dagokienez. Kiel-en, muskuiluak udaberriko fitoplanktonaren loraldian eta POC-en bigarren gailurretan lagindu ziren, eta neurri txikiagoan udazkeneko muskuilu bilketa baino denbora pixka bat lehenago eman zen produkzio primarioaren emendioa. Kiel-en udako laginketa aldian elikagaien hornidura maila baxuenean zegoen. Tvärminne-n, Chl-a eta POC kontzentrazioak maila altuetan zeuden muskuiluak udaberrian bildu zirenean, urteko fitoplanktonaren eta karbono organikoaren lehen loraldiarekin bat eginez. Ondoren, elikagaien hornidura egonkor mantendu zen udarara arte. Udazkenean

Tvärminne-ko muskuiluak elikadura egoera nahiko onean zeuden, eta horrek adieraz dezake Finlandiako golkoan (Raateoja et al., 2011) udako zianobakterioen loraldi kaltegarriek ez zutela asaldura fisiologiko handirik sortu urte osoko eskalan.

Ehunetako PAH kontzentrazioa orokorrean baxua izan zen, eta detekzio mugatik gorako konposatuak, kasu guztietan, Itsaso Baltikorako ontzat jotzen diren balioen azpitik zeuden (HELCOM, 2018). Kiel-en udazkenean lagindutako muskuiluen 15 PAH-en batura gainontzeko taldeetakoa baino nabarmen handiagoa izan arren, ezin da kezka gunetzat hartu (Turja et al., 2015). Ehun bigunetan aztarna metalen kontzentrazioa ere baxua zen. Itsaso Baltikoan lehentasunezko substantziatzat hartzen diren hiru metalak (kadmioa, beruna eta merkurioa) Itsaso Baltikoko GES atalasearen balioen azpitik edo zertxobait gainetik zeuden (HELCOM, 2017). Ondorioz, laginketa-estazioetako kutsatzaile maila baxuek iradokitzen dute biomarkatzaileetan erregistratutako aldakortasunak ez zuela kutsatzaileen eragin handirik izan, hemen eztabaidatutako beste faktore batzuena baizik.

Ikerketa honetan, bi tokietako ADG zelula dentsitaterik handiena udazkenean aurkitu zen errutea gertatu ostean, eta hori aurrekoarekin bat dator. Udazkenean bi lekuetan ADG zelulen maila handiagoak neurtu izanak adierazten du muskuiluek erreserba energia metatu behar dutela neguko elikadura baxuko aldirako, iparraldeko Itsaso Baltikoan hegoaldean baino gehiago irauten duenak.

Tokien arteko muskuiluen fase gametogenikoen desberdintasunak osasun egoeraren ebaluazioan erabilitako biomarkatzaileei eragin diezaieke (Cuevas et al., 2015). Kiel-en udaberriko loraldia otsailaren erdialdetik apirilaren hasiera bitartean gerta daiteke (Wasmund et al., 2008), eta hori koherentea da lan honetan antzemandako errute aldiarekin (ziurrenik martxoa edo apirilaren hasieran). Tvärminne-n, berriz, muskuiluek udan eta udazkenean errun zuten; lehenengo errute aldia loraldiarekin azaldu daiteke eta bigarrena udazkeneko loraldi posible batekin lotu liteke. Beraz, bi guneetan aldi berean lagintzen diren muskuiluen egoera fisiologikoa nahiko ezberdina izatea posible da. Hori dela eta, loraldien garaia aldatzen denean ugaltzearen hasiera erregulatzen duela ondorioztatu daiteke, eta, hortaz, biomarkatzaileak erabiliz muskuiluen osasun egoera baloratzeko orduan, tokiko baldintzak arreta handiz hartu behar dira kontuan interpretazio okerrak ekiditeko.

Biomarkatzaile biokimikoek urtaro eredu desberdinak erakutsi zituzten neurtutako jarduera entzimatikoaren arabera. Ezberdintasun esanguratsu bakarra zakatzetan AChE jardueran ikusi zen udaberriaren eta beste bi laginketa urtaroen artean, bi laginketa tokietan. Aurretik deskribatu da tenperatura dela AChE-ren jardueran eragiten duen faktore nagusia (Bocquené eta Galgani, 1998), eta, ondorioz, udan AChE-ren jardueraren igoera uraren tenperaturaren igoerarekin erlazionatuta egon zitekeen. Liseri-guruineko CAT eta GST jarduerak loraldietan eskuratutako elikagaiak eta muskuiluen ugaltze zikloak baldintzatuta zeudela ziruditen, jarduera gailurrak errute urtaroekin bat zetozelako (udaberria Kiel-en eta uda Tvärminne-n). Udazkenean errutea gertatu arren Tvärminne-ko muskuiluetan CAT eta GST jarduera-maila oso altua ez zen izan, hau liseri-jarduera nahiko baxuagoarekin lotuta egon liteke. Deskribatu da entzimen jarduera antioxidatzailearen aldakortasuna egoera metaboliko aldakor batekin lotuta egon daitekeela, ziurrenik tenperaturak, gonaden heltzeak eta elikagaien erabilgarritasunak eraginda(Regoli, 1998; Vidal et al., 2002). Leiniö eta Lehtonen-ek (2005), lan honetako emaitzekin bat etorriz, Itsaso Baltikoko muskuiluen liseri-guruinean CAT eta GST jardueraren emendioa ere deskribatu zuten lehen adierazitako faktoreekin, tenperaturarekin izan ezik. Gainera, lehenagoko ikerketek ingurumen faktoreek hegoaldeko Itsaso Baltikoko biomarkatzaile hauen erantzunean duten eragina deskribatu dute (Kopecka et al., 2006), lan honetan deskribatutako aldakortasunarekin bat etorriz.

Bi ikerketa eremuetan, LPO maila altuagoak deskribatu ziren udazkenean beste bi laginketa garaiekin alderatuta. Aurretik egindako ikerketek lipido poliasegabeetan aberatsak diren mintz zelularrak erradikal askeekin erreakzionatzeko gai direla frogatu dute (Shaw et al., 2004); horrela, neguko tenperatura hotzetara egokitutako mintz zelular jariakorragoak (gantz-azido poliasegabe gehiagoduna) kalte oxidatiboarekiko sentikortasun handiagoa azal dezake udazkenean bildutako muskuiluetan. Mintz zelularreko lipidoen aldaketak hotzetarako egokitzapen gisa deskribatu izan dira aldez aurretik Itsaso Baltikoko beste itsas organismo batzuetan, hala nola anfipodoetan (Lahdes et al., 2010), nahiz eta ideia hau sakonago ikertu beharko litzatekeen. Ondoriozta daiteke biomarkatzaile biokimikoen neurketak urtaroko aldakortasun handia duela ingurumen faktoreek eraginda, eta funtsezkoa dela urtaro eta toki

desberdinetako erreferentzia mailei buruzko ezagutza biltzea, kutsatzaileek eragindako efektu biologikoak ebaluatzeko biomarkatzaileak era egokian erabili ahal izateko.

Tvärminne-n udaberrian bildutako muskuiluetan lipofuszinen bolumen dentsitate handia neguan zehar luzatu zen, liseriketa estresaren seinale izan litekeena, lekuko izotz estalkia eta eguzki argi ezarekin batera. Neguan izotz estalkipean bizi diren muskuiluetan estres nutrizionala aurretik deskribatu da (Hatcher et al., 1997) eta, gainera, udazkenean neurtutako lipidoen peroxidazio maila altuagoa muskuiluek neguko baldintza gogorretan bizirauteko beharrezko dituzten estrategien adierazle izan daiteke, zeinek izotz estaldura aldian kalte oxidatiboa jasateko gaitasun handiagoa ekarriko bailukeen. Gainera, udaberriko fitoplanktonaren lehen loraldian izotza hautsi ondoren liseri-jarduera handia izateak liseri-zelulen barruan lipofuszinen presentzia handiagoa ekar zezakeen. Kiel-en udazkenean lagindutako muskuiluetan lipofuszina metaketa handia bat dator neurtutako egitura aldaketa lisosomikoekin. Gainera, udazkenean lipidoen peroxidazio maila altua gune honetako lipofuszina metaketa maila altuen arrazoia izan daiteke. Tvärminne-n udazkenean antzeko lipidoen peroxidazio mailak neurtu baziren ere, lipofuszinen metaketak eta egitura aldaketa lisosomikoak ez dute joera bera jarraitzen. Desadostasun hori kontuan hartu behar da biomarkatzaileen erantzunaren interpretazioari dagokionez nahaste faktore posibleak saihesteko. Ikerketa honetan muskuiluen ehun bigunetan PAH maila baxuak soilik neurtu direnez, lipido neutroen metaketaren aldaketak aldaketa fisiologiko edota ekologikoekin erlazionatuta egotea espero daiteke. Liseri-guruineko lipidoen edukia ez dago ugaltze zikloarekin hain lotuta, mantuko lipidoak ez bezala (Moukrim et al., 2008). Beraz, ikerketaren emaitzen arabera, liseri-guruineko lipido neutroen edukia kutsatzaile organikoen biomarkatzaile baliotsua izan daiteke, ez baitzuen aldakortasun handirik erakutsi urtaro desberdinetan ikertutako lekuetan.

Zoritxarrez, aldaketa lisosomikoak ezin izan ziren fidagarritasunez neurtu bi tokietan udaberrian. Egitura aldaketa lisosomikoen neurketen kasuan, Kiel-eko muskuiluek liseri-zeluletan ezohiko orban ilunak aurkeztu zituzten β-glukoronidasa tindaketa egin ondoren, eta ezinezkoa izan zen populazio lisosomikoen neurketa zuzena egitea. Tvärminne-ko muskuiluek forma pikordun orban hedatuak zituzten liseri-zeluletan, eta, beraz, neurketa estereologikoak ez ziren fidagarriak. Tvärminne-ko

muskuiluetan parametro hauek neurtu ez izana, ziurrenik, zelula barneko lipofuszina eta/edo alga eduki handiarekin lotuta dago, horrek jarduera entzimatikoaren tindaketa histokimikoa oztopatu eta neurketa engainagarriak eragin ditzakeelako (Mújica et al., 2015a). Kiel-eko muskuiluen kasuan, orban ilunen arrazoiak ez daude argi. Neurgarriak izan ziren laginetako egonkortasun lisosomikoaren probaren emaitzak antzekoak izan ziren bai urtaro bai laginketa guneen artean, eta Kantauri itsasoko egoera ekologiko egokiko muskuiluetan deskribatutako balioetatik hurbil zeuden (> 20 min) (Marigómez et al., 2006). Egitura aldaketa lisosomikoei dagokienez, desberdintasun esanguratsu bakarrak Kiel-eko udazkenean lagindutako muskuiluetan aurkitu ziren, bolumen dentsitatea Tvärminne-ko muskuiluetan baino handiagoa izan zen denboraldi berean . Kiel-eko udazkenean lagindutako muskuiluen azalera/bolumen erlazioa udan Kiel-eko muskuiluetan eta udazkeneko Tvärminne-ko muskuiluetan baino txikiagoa zen. Handitze lisosomikoa muskuiluak kutsatzaile ezberdinen eraginpean daudenean gerta daiteke (Marigómez et al., 2005) edo faktore natural batzuk aldatzen direnean (Izagirre et al., 2008). Lan honetan, kutsatzaileen eragin posibleak oso mugatuak izan daitezke tokiko kutsadura kimikoaren hurbilpen gisa erabiltzen diren metalen eta PAHen ehunetako kontzentrazio baxua kontuan hartuta. Udazkenean Kiel-en bildutako muskuiluetan deskribatutako handitze lisosomikoa bat dator lipidoen peroxidazioa eta lipofuszina metaketa bezalako erantzunekin.

Ehun mailako biomarkatzaileek joera desberdinak erakutsi zituzten haien artean. Esaterako, Vv_{BAS}-ek ez zuen urtaro aldakortasunik erakutsi, nahiz eta udaberrian eta udan tokien artean alde handiak aurkitu ziren. Lan honetako Vv_{BAS} altuenak estres apur bat iradoki dezake Kantauri itsasoko muskuiluetan definitutako oinarri balioekin alderatuz gero (0,1 µm3/µm3) (Marigómez et al., 2006, Garmendia et al., 2010). Kiel-en Vv_{BAS} balio altuena errute aldia baino lehen erregistratu zen, eta igoera hori estres iragankor gisa azal zitekeen, baina horrek ez du azaltzen Kiel-eko udako balioak Tvärminne-koak baino handiagoak izatea, non muskuiluak errute aldian baitzeuden. Dirudienez, Kiel-eko muskuiluetan Vv_{BAS}-en aldakortasun naturalak Tvärminne-n baino oinarrizko balio handiagoak erakusten ditu, estres kimikoen eraginak ebaluatzeko orduan kontuan hartu beharrekoa dena.

Ikerketa honetan, Kiel-en ez da CTD-ren urtaro desberdintasun esanguratsurik aurkitu, baina Tvärminne-n udan eta udazkenean (bertako errute aldiak) balio nabarmen handiagoak erregistratu ziren. Moluskuetan, CTD-ren balio altuek liseri-guruinaren osotasunaren galera adierazten dute, estres kimiko batek edo elikadura egoera txarrak eraginda (Mújica et al., 2015b, Benito et al., 2017). Beharbada izotza hautsi ondoren eta lehen algen loraldiarekin batera muskuiluek elikagai kopuru handiena asimilatu behar dute beren energia erreserbak betetzeko, Ipar Atlantikoko muskuiluetan deskribatzen den bezala (Hatcher et al., 1997). Ondorioz, izotza hautsi ondoren gonadaren heltze azkarrak inbertsio energetiko handiak eskatuko lituzke muturreko inguruneetan, Finlandiako golkoan esaterako, errute sasoietan ikusitako liseri-albeoloen atrofia eta CTD balio handiagoak azal ditzaketenak. Kiel-eko muskuiluetan epitelioaren mehetze eta CTD ratioari dagokionez, ugaltze zikloak gutxiago eragiten duela dirudi, hego mendebaldeko Itsaso Baltikoko elikagaien eskuragarritasun egonkorragoarekin lotuta egon zitekeena, eta, ondorioz, ugalketaren inbertsioarekin erlazionatutako truke energetikoa Tvärminnen baino txikiagoa izan liteke (Larsson et al., 2018). Badirudi Finlandiako Golkoko muskuiluek neguan gosete aldi luzea igarotzen dutela, izotz estaldura eta argi aldi laburrak eragindako ekoizpen primario oso baxuaren ondorioz.

Infiltrazio hemozitikoa ohikoa zen Tvärminne-ko muskuiluetan urtaro guztietan eta neurri txikiagoan Kiel-eko muskuiluetan udan. Aurreko lanek infiltrazio hemozitikoa faktore ekologiko eta fisiologikoekin lotu zuten, besteak beste, gosea, ugaltzeko estresa eta parasitismoarekin (Couch, 1985; Garmendia et al., 2011), baita Finlandiako Golkoan ere (Sunila, 1987). Horrenbestez, Tvärminne-ko muskuiluen infiltrazio hemozitikoen (erantzun immunea) prebalentzia altua inguruko gazitasun baxuarekin eta elikagaien hornikuntza ezegonkorrarekin lotuta egon liteke, lehen esan den bezala (Malagoli et al., 2007, Bussell et al., 2008). , Höher et al., 2012). Elikagaiak ugariagoak direnean ere (Cl-a eta POC altuak) errute esfortzuak estres nabarmena eragiten du, biomarkatzaileen erantzun ezberdinek frogatzen dutenez, eta horrek infiltrazio hemozitikoen maila altua hein batean azal dezake. Tvärminne-n udaberrian bildutako muskuiluen kasuan, infiltrazio hemozitikoen prebalentzia handia neguko gose aldi luzearen ondorio izan daiteke. Kiel-eko muskuiluetan, infiltrazio hemozitikoen prebalentzia handiagoa ziurrenik parasito digeneoaren prebalentzia apur bat handiagoarekin eta udaberriko

loraldiaren eta ugalketaren ondoren elikagaien eskuragarritasun txikiagoarekin lotzen da. Liseri-guruinean zelula arreen infiltrazioa liseri-traktuko epitelioan ikusi zen, non melanizatutako hemozitoak epitelioa zeharkatzen baitzuten diapedesi bidez. Diapedesi tasarik handiena Tvärminne-n udaberrian lagindutako muskuiluetan gertatu zen eta lipofuszina pikorrak/mikroalgak ezabatzeko mekanismo posible bat izan liteke (Galimany et al., 2008), Ipar Itsaso Baltikoko aurreko emaitzekin koherentea dena (Lehtonen, 1989). Gonada folikuluetan zelula arreak egotea eta obozitoen atresia gametogenesian zehar abian diren autolisi eta birxurgatze prozesuen adierazle dira, eta ingurune baldintzak (elikagaien eskuragarritasuna, tenperatura eta kutsadura) gametoen heltzearen ondoren ugaltzeko desegoki bihurtzen direnean gerta daiteke (Newell, 1989; Suárez-Alonso et al., 2005; Smolarz et al., 2017). Zelula arreen infiltrazioak mailarik altuenak Tvärminne-n udan lagindutako muskuiluetan aurkitu ziren, eta, ondoren, gune berean udaberrian bildutakoak izan ziren. Bi fenomenoek (zelula arreen infiltrazio handiak eta atresiaren prebalentziak) Tvärminne-ko muskuiluak bizirik irauteko beharrezkoa den estrategia energetiko zorrotza adieraz dezakete. Gainera, atresia udaberritik udazkenera gutxitzen da eta errutea udan eta udazkenean gertatzen da Kiel-eko muskuiluetan bezala, ziurrenik errute-aldien ondoren gameto helduen kopuru txikiagoarekin lotuta dagoena.

Granulozitomak oklusio baskularren ondoriozko hanturazko erantzunak dira (Lowe eta Moore, 1979), kutsadura kronikoarekin eta parasito metazerkarioen presentziarekin lotuta egon direnak (Garmendia et al., 2011). Lan honetan, granulozitomak Tvärminne-n udaberrian lagindutako muskuiluetan bakarrik aurkitu dira (%20). Badirudi patologia hau ez dela ehunetako kutsatzaileen kontzentrazioen (baxua) eta parasitoen prebalentziarengatik (altua MPX zelula barneko ziliatuentzat bakarrik). Beste arrazoi bat, Rasmusenek (1986) adierazi bezala, leukozito pikortsuez osatutako granulozitomen eta infekzio birikoen arteko erlazioa da. Horren arabera, udaberrian granulozitomak aurkitu ziren Tvärminne-n, non immunitate sistema deprimituta egon baitzitekeen, eta neguko gosealdiaren ondoren infekzioak izateko sentikortasun handiagoa espero daitekeen. Butt et al. (2007)-ek frogatu zuten goseteak jarduera immunologikoa arriskuan jartzen duela Sydneyko harkaitz-ostretan (*Saccostrea glomerata*). Hala ere, ikerketa honetan tamaina txikiko granulozitoma bakarra aurkitu

da banako bakoitzeko, beraz, lesio hauek ez dira oso arriskutsuak izango laginketa leku nahiko garbietan finkatuta dauden muskuiluentzat.

Azkenik, hautatutako biomarkatzaileetatik kalkulatutako IBR/n indizea erlazionatuta dago toki bakoitzeko errute sasoiekin, nahiz eta neurtutako erantzunak desberdinak izan. Udaberrian, IBR/n balio altuena Kiel-eko muskuiluen artean izan zen, eta horrek erruteak sortutako estres egoera bat iradokitzen du. Alderantziz, IBR/n baliorik baxuena Tvärminne-tik udaberrian bildutako muskuiluetan ikusi zen, oraindik errutea gertatzear zenean. Errutea Tvärminne-n gertatu zen bi aldietan eta Kiel-en errutearen ondorengo bi aldiek antzeko estres balio integratuak erakutsi zituzten.

ONDORIOAK

Urtaroen joera ezberdineko tokiak alderatzeko biomarkatzaileen erantzunak neurtzean, posible da muskuiluak ingurune-baldintza ezberdinen eraginpean egon daitezkeela. Eragindako parametroen artean, gametoen heltzeak eta erruteak berebiziko garrantzia dute, neurketa biokimikoak (katalasa eta GST), histologikoak (CTD eta atrofia) eta alterazio histopatologikoak eragiten baitituzte. Gametoen garapenean elikagaien hornikuntzaren eragina argi eta garbi dagoenez, zelai ikerketetan aztertutako tokietako baldintza fisiko-kimikoak (lan honetan sateliteen datuek adierazten duten moduan) identifikatzearen garrantzia argia da. Zehatzago esanda, Itsaso Baltiko barneko eskualde ezberdinek ezaugarri ozeanografiko eta klimatiko desberdinak dituzte, eremu epel/subartiko honetan askoz aldakorragoak direnak eremu epeletan baino. Udazkenean ikusitako ADG mailen igoera Itsaso Baltikoan negurako erreserba materiala metatzeko beharrarekin lotuta egon liteke eta, ondorioz, lipidoen peroxidazioaren, lipofuszinen metaketa eta lisosomen handitzea probabilitate handiagoa ekarriko lukeena. Tvärminne-n, negu luzeak (izotz estaldurarekin batera) udaberrian susperraldi somatikoan zentratzen zen muskuiluen urte osoko estrategian eragina izan zuen (elikadura jarduera handia, lipofuszina eduki handia eta atrofia indize eta CTD ratioa baxua eragin zuen). Ingurugiro faktoreen urtaroen gorabeherek lipido neutroen metaketa nabarmen eragin ez zutenez, biomarkatzaile hau gomendagarria da Itsaso Baltikoko muskuiluetan kutsatzaile organikoen eraginen jarraipena egiteko. Lan honetan aztertutako gainontzeko biomarkatzaileentzat garrantzitsua da urtaroen efektuak eta

Itsaso Baltikoko leku desberdinetako biomarkatzaileen oinarri balioetan eragina duten ingurumen faktore ezberdin eta aldakorren berri ematea. Gune bakoitzean aldagai ekologikoen eta baldintza fisiko kimikoen karakterizazioa funtsezkoa da Itsaso Baltikoan kutsadura eszenatoki hipotetiko baten ondorioen ebaluazio fidagarria egiteko. Gainera, biomarkatzaileen erreferentzia-mailak eta ingurumen baldintza naturalen erantzunak ezarri behar dira.

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Chapter 2: Pilot study to determine reference levels and the responsiveness to pollution of a battery of cell and tissue level biomarkers in mussels (*Mytilus edulis*) from subarctic and arctic localities in the Norwegian Sea.

Kongresuak:

SETAC 2018: "Cellular and tissue-level biomarkers in mussels (*Mytilus edulis*) sampled in two different study areas in the Northern Atlantic" Denis Benito, Xabier Lekube, Urtzi Izagirre, Ionan Marígomez, Beñat Zaldibar, Manuel Soto. Poster presentation.

ABSTRACT

North Atlantic and Arctic Oceans contain noteworthy amount of undiscovered oil and gas reserve, the threat of oil spills is blooming and their hazardous ecological consequences are relevant to be studied. Although mussels (*Mytilus sp*.) respond clearly to contaminants, biomarkers have shown a variability due to biological and environmental changes that occur in nature. Mussels can experience biochemical, metabolic or physiological changes which might act as confounding factors altering the organisms' ability to respond to the presence of pollutants. In order to help avoiding misinterpretation of biological responses, the aim of this chapter is to reveal the effect of natural variability (latitude-related environmental factors and age of the collected animals) in the responsiveness to pollution of a battery of cell and tissue-level biomarkers in mussels of two size-classes (2-3 cm and 3.5-4.5 cm) collected in relatively non-impacted sites and potentially impacted sites as harbors and a waste water treatment plant in early autumn of 2016 in Trondheim and Tromsø. Although the battery of biomarkers used here proved to be useful to discriminate impacted and non-impacted mussel populations, some confounding factors altering the biological responses have been identified. Geographical/latitudinal factors seemed to be critical regarding the reproductive cycle, reserve material storage and the prevalence of parasites such as Gymnophallidae like trematodes. Size (age) of mussels seemed to be important regarding the physiological condition and the coping capabilities of mussels towards moderate chemical insult, although this was only detected in some biomarkers in small mussels from the port in Tromsø. On the contrary, the mussels from the reference site in Tromsø displayed general stress responses at different levels, which could be influenced by the pathogenic effect of the Gymnophallidae like trematode and by a more advanced gametogenic developmental stages compared to the mussels from Trondheim, which could lead to misinterpretation of the stress levels in those mussels. All in all, the current work serves as an anchor point both as a reference of the baseline level values of the analyzed endpoints in the studied geographical area and time of the year, and as an indication of the potential extent of the environmental confounding factors causing stress on the analyzed mussel populations.

LABURPENA

Ipar Ozeano Atlantiko eta Ozeano Artikoek aurkitu gabeko petrolio eta gas erreserba kopuru nabarmena dute, petrolio isurien mehatxua loratzen ari da eta haien ondorio ekologiko arriskutsuak aztertzea garrantzitsua da. Muskuiluek (*Mytilus sp.*) kutsatzaileei argi erantzuten dieten arren, biomarkatzaileek aldakortasuna erakutsi dute naturan gertatzen diren aldaketa biologikoen eta ekologikoen aurrean. Muskuiluek aldaketa biokimikoak, metabolikoak edo fisiologikoak jasan ditzakete, nahaste-faktore gisa joka dezaketenak organismoek kutsatzaileen presentziari erantzuteko duten gaitasuna aldatuz. Erantzun biologikoen interpretazio okerra ekiditen laguntzeko, kapitulu honen helburua aldakortasun naturalak (latitudarekin erlazionatutako ingurumen faktoreak eta bildutako animalien adina) zelula eta ehun-mailako biomarkatzaileen bateria baten kutsaduraren aurrean duen erantzun gaitasuenan sortu dezaken eragina agerian jartzea da, 2016-ko udazkenaren hasieran Trondheim eta Tromsø-n, eragin antropogenikorik gabeko eta eragin antropogenikoa duten guneetan (portuak eta hondakin-uren araztegia), bildutako bi tamaina klaseko muskuiluetan (2-3 cm eta 3,5-4,5 cm). Nahiz eta lan honetan erabilitako biomarkatzaileen bateria baliagarria izan den inpaktu antropogenikoa jasaten duten eta efektu antropogenikorik gabeko muskuilu populazioak bereizteko, erantzun biologikoak aldatzen zituzten faktore nahasgarri batzuk identifikatu dira. Faktore geografikoak/latitudinalak kritikoak izan ziren ugalketa-zikloari, erreserbako materialaren biltegiratzeari eta parasitoen prebalentziari zegokionez, adibidez, Gymnophallidae antzeko trematodoak bezalako parasitoak. Muskuiluen tamaina (adina) garrantzitsua izan zen egoera fisiologikoari eta muskuiluen irain kimiko moderatuaren aurrean aurre egiteko gaitasunei dagokienez, Tromsø-ko portuko muskuilu txikietan biomarkatzaile batzuetan bakarrik detektatu bazen ere. Aitzitik, Tromsø-ko erreferentzia guneko muskuiluek estres erantzun orokorrak izan zituzten maila ezberdinetan, eta horiek Gymnophallidae antzeko trematodoen efektu patogenoaren eta garapen gametogeniko fase aurreratuago baten (Trondheim-eko muskuiluekin alderatuta) eraginpean egon zitezkeen, muskuilu horien estres mailak gaizki interpretatzea ekar zitekeena. Oro har, lan honek lanak aingura puntu gisa balio du, bai aztertutako eremu geografikoan eta urteko garaian erabilitako biomarkatzaileen oinarri balioen erreferentzia gisa, bai ingurumen nahaste faktoreek eragin dezaketen hedaduraren adierazgarri gisa.

INTRODUCTION

Various components of marine ecosystems are at risk of being affected by elevated concentrations of contaminants released from anthropogenic sources. The concentrations of PAHs, PCBs and metals in European coastal waters are mostly decreasing where monitored, but the amount of data is scarce in some areas, including the Norwegian Sea (EEA, 2018). North Atlantic and Arctic Oceans contain noteworthy amount of undiscovered oil and gas reserves (Gautier et al., 2009), the threat of oil spills is blooming and their hazardous ecological and socio-economic consequences are relevant to be studied. Moreover, Arctic ecosystems are highly vulnerable to oil spills due to their peculiar environmental conditions (low temperature of seawater and, more remarkably, the presence of ice-cover) and remoteness. These features could modify the chemical composition of the spill products and intensify the toxicity to marine biota (Nordam et al., 2017; Word, 2014) and they can be major factors hampering clean-up operations after oil spills.

Mussels (*Mytilus sp*.) are widely used as sentinel species of marine pollution as they are ubiquitous, sessile, filter feeding organisms placed on a low trophic level, they accumulate contaminants in their tissues and allow the monitoring of the bioavailable fraction of contaminants through time along a wide geographical distribution (Bellas et al., 2014; Beyer et al., 2017; Cuevas et al., 2015; Viarengo et al., 2007). Even though they respond clearly to contaminants, biomarkers have shown a variability due to biological and environmental changes that occur in nature (Benito et al., 2019; Beyer et al., 2017; Depledge, 2009; Fernández et al., 2010; Nahrgang et al., 2013). Mussels can experience biochemical, metabolic or physiological changes which might act as confounding factors altering the organisms' ability to respond to the presence of pollutants, therefore, there is a need for deeper understanding of the biological cycles influencing them and the baseline levels of the biomarkers in question in order to correctly interpret biomarker responsiveness (Beyer et al., 2017; Nahrgang et al., 2013; Storhaug et al., 2019).

The blue mussel (*Mytilus edulis*) has a wide distribution in boreo-temperate region, in the North-Pacific, North- and Mid-Atlantic up to the Arctic Ocean (Kijewski et al., 2011). Their growth and reproduction was shown to be dependent on temperature

and on food availability (Berge et al., 2005; Beyer et al., 2017; Page and Hubbard, 1987; Sprung, 1983; Stirling and Okumuş, 1995; Storhaug et al., 2019; Thorarinsdóttir and Gunnarsson, 2003). Consequently, there can be a misinterpretation of biomarker responses due to the potential confounding factors related with age or reproductive conditions, the responsiveness of biomarkers against pollution being compromised. Hence, the need to decipher how the natural variability of these confounding factors can affect biomarker responses it is clear (Bellas et al., 2014; Beyer et al., 2017; Bignell et al., 2008; Cuevas et al., 2015).

This study aims to reveal the effect of natural variability (latitude-related environmental factors and age of the collected animals) in the responsiveness of a battery of selected cell and tissue-level biomarkers towards pollution in mussels of two size-classes collected in relatively non-impacted sites and potentially impacted sites as harbors and a waste water treatment plant in early autumn of 2016 in Trondheim and Tromsø.

MATERIAL AND METHODS

Sampling, sampling processing and biomarker analysis

The collection of mussels (*Mytilus edulis*) consisted in three sampling sites in Trondheim fiord including an allegedly non-impacted site used as a reference site and a mussel depuration facility in Rissa (63.561753, 9.899776) on the 18/10/2016, a harbour in Trondheim (63.442692, 10.425494) on the 17/10/2016 and a rocky beach in the vicinity of a WWTP effluent in Trondheim (63.444867, 10.341331) on the 19/10/2016. Sampling sites in Tromsø included a rocky beach in an allegedly non-impacted site used as a reference site (69.642089, 18.94639) and harbour (69.654177, 18.968459), mussels in both sampling sites were collected on the 20/10/2016.

Mussels were sampled from the first meter of the lower intertidal zone and were taken to the laboratory in air at ambient temperature, dissection was carried out immediately. In each dissection, transversal slices including mantle, gills and digestive gland were performed in 20 mussels for histopathological analysis and tissue-level

biomarkers. Additional samples of whole mussels were frozen for chemical analysis of soft tissues.

Sample processing, adipogranular cell index, gamete development, cellular biomarkers, tissue-level biomarkers and histopathology were performed as described in chapter 1.

Statistical analysis

Statistical analysis was carried out with the aid of the SPSS/PC+ statistical package V.24 (SPSS Inc., Microsoft Co.). For the quantitative data which passed normality test, one-way ANOVA and subsequent Duncan's post-hoc test for multiple comparisons between pairs of mean values was applied ($p < 0.05$). For the semiquantitative results obtained and the quantitative results which did not pass normality test, non-parametric Kruskal-Wallis ANOVA tests were carried out comparing the distribution using pairwise comparison ($p<0.05$).

Chemical Analysis of PAHs and metals

The analysis of mussel tissues was carried out following the method described by Navarro et al. (2009). Briefly, freeze-dried samples (0.25-0.5 g) were accurately weighed in the Falcon vessel and a known amount of deuterated PAHs solution was spiked as internal standard and 5 ml of acetone. The PAHs extraction was performed by an ultrasound homogeniser SONOPULS HD 2070 (Bandelin electronic GMBH & Co. KG, Berlin, Germany) at 45% of power (max power 70 W) and for 2 min. The extracts were filtered by Millex® HV PVDF 0.45 µm (Millipore, Carrigtwohill, Ireland) and concentrated to ∼0.5 mL under a nitrogen stream (TurboVap LV, Zymark, Barcelona, Spain) after the addition of ∼1 mL of iso-octane. The concentrated extracts were cleaned with 1 g Florisil cartridges previously conditioned with n-hexane, and eluted with 25 mL of n-hexane:toluene 75:25 mixture. Subsequently, they were concentrated to dryness, redissolved in iso-octane and kept at −20 °C in the dark until the GC-MS analysis.

The PAH analysis was carried out on a 6890N Agilent gas chromatograph coupled to a 5973N Agilent mass spectrometer (Agilent Technologies, Avondale, USA) with a

7683 Series Agilent autosampler. The sample (2 µL) was injected in the splitless mode at 270 °C into a (30 m × 0.25 mm × 0.25 µm) HP-5 capillary column. The temperature program used for the chromatographic separation was as follows: 60 °C for 2 min, temperature increase at 10 °C min−1 to 290 °C where it was finally held for 10 min. The carrier gas was helium (C-50) and was kept at a constant flux of 1.5 mL min−1. The mass spectrometer was operated in the electron impact ionisation mode and the energy of the electrons was kept at 70 eV. The interface was kept at 300 °C and the ionisation source and the quadrupole at 230 °C and 150 °C, respectively. Measurements were performed in the selected ion monitoring (SIM) mode.

Chemical analysis of metals was performed as described in chapter 1

RESULTS

Chemical burden in soft tissues

The distribution of light and heavy PAHs in the soft tissues are plotted in Figure 1 together with the ratio Fluoranthene/(Fluoranthene +Pyrene) (F/(F+P). As can be seen, the highest concentrations of PAHs are linked to the most polluted sites (i.e. small mussels from the port in Trondheim and mussels of both sizes from the port in Tromsø) and the lowest to the farm and reference sites. Additionally, higher concentrations of heavy PAHs (H-PAH, 4 or more aromatic rings) than the light ones (L-PAH, less than 4 aromatic rings) are observed in all the sites except in the farm in Trondheim, which belongs to the one with the lowest total concentration.

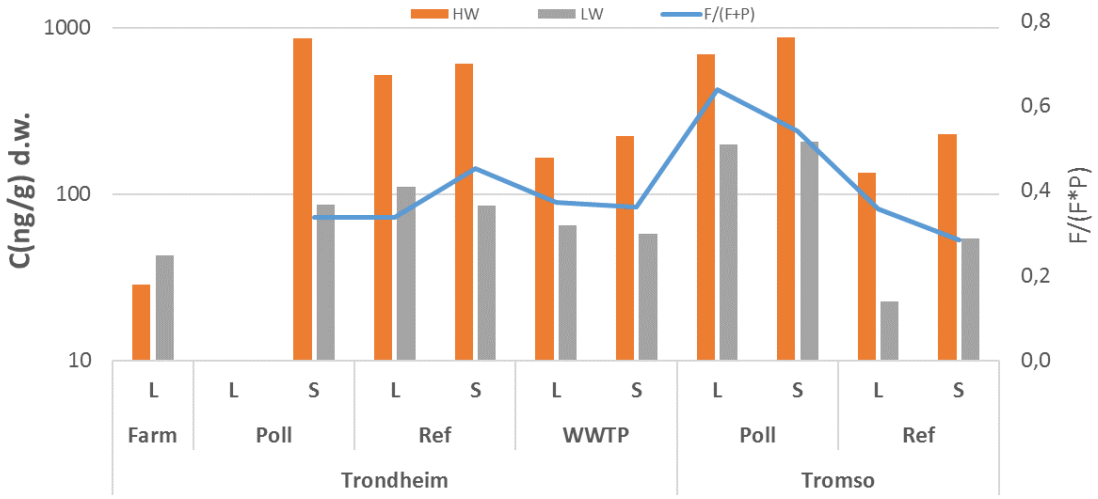

Figure 1: Plot of the light and heavy PAHs and the fluoranthene-pyrene (F/(F+P) ratios for the mussels tissues. HW: Heavy molecular weight. LW: Light molecular weight. L: Large mussels. S: Small mussels.

In order to provide a broader view of the distribution of PAHs and heavy metals (supplementary materials), a principal component analysis (PCA) was carried out in The Unscrambler X (v 10.5.1, CAMO, Norway) program. As shown in Figure 2, based on centered and scaled values, the first two PCs explained up to 72% of the total variance, and the PC1-PC2 score plot reveals that animals from both ports are distant from the reference sites and the WWTP. Mussels from both ports presented predominant influence of different metals and PAHs. Mussels from the farm in Trondheim presented less influence of contaminants than the rest of the groups. No relevant differences were found among mussels of different size from the same sampling site.

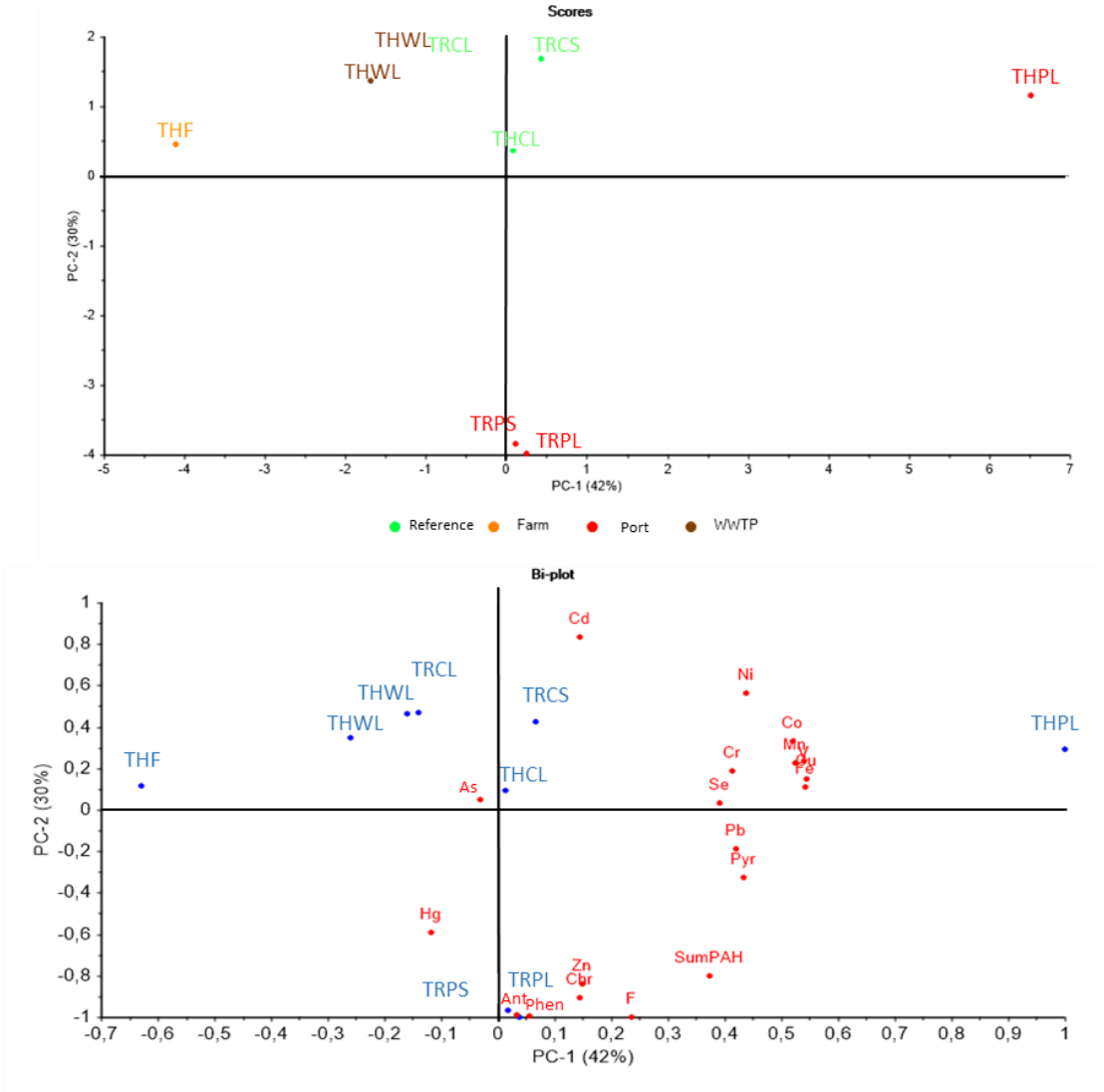

Figure 2: Plot of the 2 Principal Component Analysis measurements explaining the 72% of the total variation of the sampled groups caused by the concentration of pollutants in soft tissues of mussels. TH: Trondheim, TR: Tromsø, C: Reference site, F: Farm, W: WWTP, P: Port.

Adipogranular cell index

ADG index (Fig. 3) presented high interindividual variability being significant low levels detected only for small mussels sampled in the harbors in Trondheim and Tromsø.

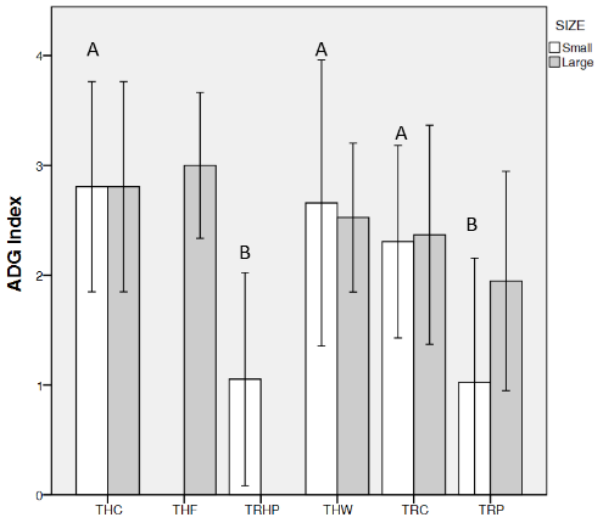

Figure 3: Adipogranular cell index in mantle tissue. TH: Trondheim, TR: Tromsø, C: Reference site, F: Farm, W: WWTP, P: Port. Letters mean statistical differences.

Gamete developmental stages

Gamete developmental stages (Fig. 4) showed differences between the mussels sampled in Trondheim and Tromsø. However, no relevant changes were detected when comparing mussel sizes or mussels from reference sites and harbors in the same locality except in the WWTP in Trondheim. Mussels from Trondheim (reference site and port) were mostly in early gametogenesis while the ones from the WWTP presented advanced gametogenesis. Mussels from Tromsø were predominantly in advanced gametogenesis.

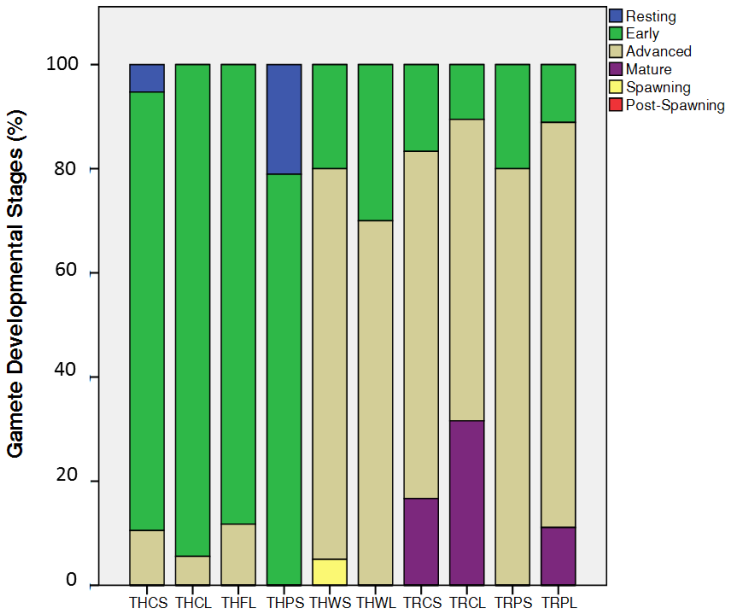

Figure 4: Gamete developmental stages (%). TH: Trondheim, TR: Tromsø, C: Reference site, F: Farm, W: WWTP, P: Port, S: Small, L: Large.

Vv_{LPF} in the digestive cells of mussels (Fig. 5A) presented great interindividual variability in the majority of the sampled groups. Large mussels from the reference site and the farm and small mussels from the harbor in Trondheim showed significantly higher values than mussels from the two sizes from the clean site in Tromsø.

Vv_{NL} (Fig. 5B) showed geographical differences, hence mussels form Trondheim exhibited significantly lower values than mussels from Tromsø. Exceptions were found in small mussels from the WWTP in Trondheim and large mussels from the harbor in Tromsø which showed intermediate values.

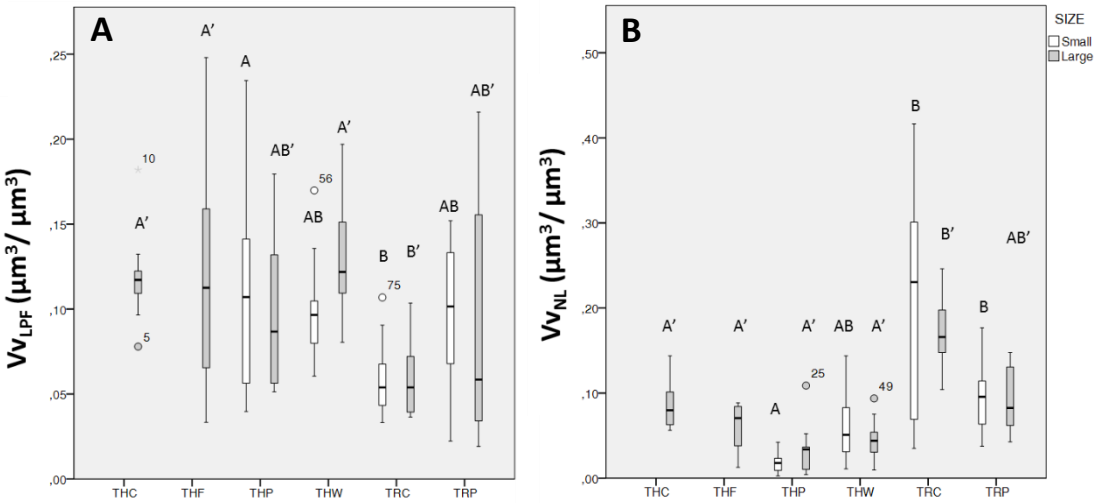

Figure 5: Box plots showing A: lipofuscin volume density (Vv_{LPF}) and B: Neutral lipid volume density (Vv_{NL}). TH: Trondheim, TR: Tromsø, C: Reference site, F: Farm, W: WWTP, P: Port. Letters mean statistical differences.

Mussels from reference sites in Trondheim and Tromsø exhibited the highest LP values (Fig. 6) ranging 15-18 min. Significant differences were observed in both size classes of mussels from the harbors in Trondheim and Tromsø with the lowest labilisation periods recorded. Mussels from the WWTP in Trondheim displayed intermediate LP values.

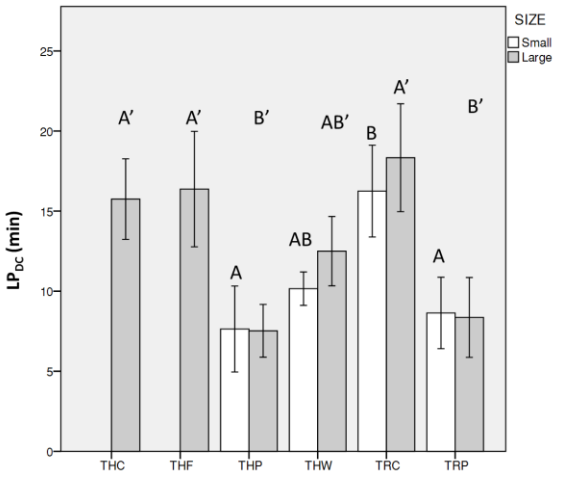

Figure 6: Labilisation periods (LP, min) measured in Lysosomal Membrane Stability test. TH: Trondheim, TR: Tromsø, C: Reference site, F: Farm, W: WWTP, P: Port. Letters mean statistical differences.

S/V_{LYS} (Fig. 7B) did not show significant differences among small mussels from different sampling sites. Significantly higher S/V_{LYS} was measured in the large mussels from the harbor in Trondheim, while significantly lower values were seen in the large mussels from the reference sites in Trondheim and Tromsø.

Mussels of both size classes from the harbor in Trondheim showed significantly higher NV_{LYS} values (Fig. 7C).

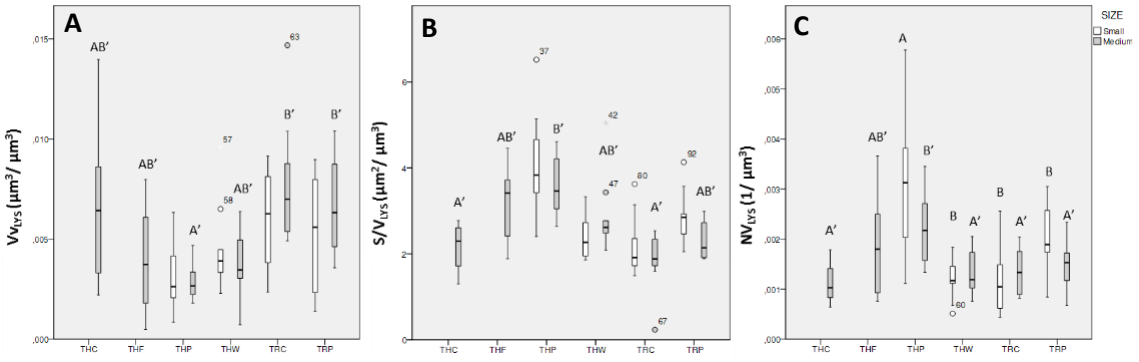

Figure 7: A, lysosomal volume density (Vv_{LYS}); B, lysosomal surface/volume ratio (S/V_{LYS}) and C, lysosomal numeric density (NV_{LYS}). TH: Trondheim, TR: Tromsø, C: Reference site, F: Farm, W: WWTP, P: Port. Letters mean statistical differences.

Vv_{BAS} levels (Fig. 8A) were significantly higher in the small mussels from both harbors in Trondheim and Tromsø. Large mussels from the WWTP in Trondheim displayed significantly higher Vv_{BAS} values than the ones from the farm in Trondheim. In addition, small mussels from the harbor in Tromsø showed significantly higher Vv_{BAS} values than the large mussels in the same sampling site.

Atrophy index (Fig. 8B) was higher in small mussels from the harbor in Trondheim than the ones in the same size class from the reference site in the same locality. Large mussels from both sampling sites in Tromsø showed higher atrophy index values than the large mussels from the reference site in Trondheim.

CTD ratio (Fig. 8C) was significantly lower in the small mussels from the reference site in Trondheim. No differences in CTD ratio were found in large mussels.

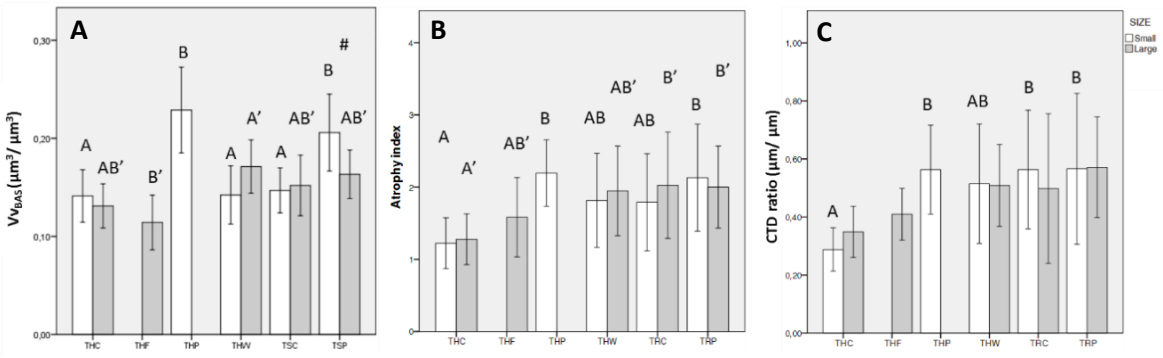

Figure 8: Graphs showing mean values of A, basophilic cell volume density (Vv_{BAS}); B, atrophy index and C, connective to digestive tissue (CTD) ratio. TH: Trondheim, TR: Tromsø, C: Reference site, F: Farm, W: WWTP, P: Port. Letters mean statistical differences.

Parasitic burden (Table 1) was low in all sampling groups from Trondheim except in mussels of both sizes sampled in the WWTP where the prevalence of Renicolidae like trematodes was 25% in the small mussels and 45% in large mussels. Mussels sampled in Tromsø showed higher prevalence of trematode (Gymnophallidae and Renicolidae) infection. Small mussels from the reference site displayed a prevalence of 20% of Gymnophallidae like parasites and a prevalence of 10% regarding Renicolidae like trematodes. Large mussels from the reference site in Tromsø presented a prevalence of 9.53% of Gymnophallidae like trematodes while the prevalence of Renicolidae like parasites was 14.29%. Small mussels from the harbor in Tromsø showed a

Gymnophallidae like parasites infection prevalence of 20% while large mussels from the same site presented a prevalence of Gymnophallidae like trematodes of 15%.

Among the pathological alterations (Table 1) considered inflammatory reactions, brown cell infiltration in the digestive gland was only found in large mussels from the WWTP in Trondheim (prevalence of 10%) and in the small mussels from the harbor in Tromsø (prevalence of 15%). Brown cell infiltration in gonad was present in large mussels from the farm (5%) and in small mussels from the WWTP (15%) in Trondheim .While in Tromsø, the 15% of small mussels from the reference site, the 5% of the small mussels from the harbor and the 25% of the large mussels from the harbor presented infiltration of brown cells in the gonad. Atresia of the oocytes showed low prevalence in all the mussels from the reference site and the harbor in Trondheim (below 13%). On the contrary both size classes in the WWTP in Trondheim presented a prevalence of 60%. In Tromsø the atresia of the oocytes was very prevalent all groups ranging between 70% and 92%. The presence of granulocytomas was very low in Trondheim, only being found in 5% of the small mussels from the reference site and in the large mussels from the WWTP. In mussels sampled in Tromsø granulocytomas were highly prevalent in both size classes and sampling sites. Small mussels from the reference site in Tromsø showed a prevalence of 10% while the one quantified in large mussels from the same site was 25%. Small mussels from the harbor presented a granulocytoma prevalence of 15% while large mussels exhibited a 10%. The prevalence of haemocytic infiltrations was relatively low in the reference site and the farm in Trondheim while mussels from both size-classes from the WWTP presented an infiltration prevalence of 40%. In Tromsø small mussels from the reference site showed a prevalence of 55% while large mussels in the same site had a prevalence of 50%. Both sizes of mussels sampled in the harbor presented a haemocytic infiltration prevalence of 50%.

Table 4: Prevalence (%) of RLO/CLO, Gymnophallidae and Renicolidae trematodes, Mytilicola sp., brown cell infiltration in digestive gland and gonad, atresia of oocytes, granulocytoma and haemocytic infiltration. DG: digestive gland, G: gonad.

			RLO/ CLO	Gymnophallidae	Renicolidae	Mytilicola sp.	Brown cell (DG)	Brown Cell (G)	Atresia	Granulocytoma	Haemocytic inf.
Trondheim	Reference	Small	0	0	0	5	0	0	0	5	5
		Large	0	0	0	5.56	0	0	5.56	0	16.67
	Farm	Large	0	0	0	0	0	5	0	0	15
	Port	Small	5.26	0	0	5.26	0	0	5.26	0	47.37
	WWTP	Small	5	0	25	0	0	15	30	0	40
		Large	0	0	45	0	10	0	30	5	40
Tromso	Reference	Small	5	20	10	0	0	15	55	10	55
		Large	0	9.53	14.29	0	0	0	45	25	50
	Port	Small	0	20	0	0	15	5	40	15	50
		Large	0	15	0	0	0	25	35	10	50

DISCUSSION

The present study provides a first approach to revealing the responsiveness of a battery of selected biomarkers in mussels sampled in two localities (one in subarctic and the other arctic latitudes) in the Norwegian Sea to assess their environmental health status regarding the variability caused by latitude-related environmental factors and physiological (age and reproductive stage) status. The knowledge of the influence of these confounding factors is crucial to correctly assess the impact caused by a potential environmental disaster (e.g. oil spill) in cold waters and to design efficient biomonitoring programs in latitudes with marked environmental differences.

According to Zhang et al (Zhang et al., 2008), the low light-to-high PAH ratio (<<1.0, except for the sample pointed before) suggest a pyrogenic source. In addition to this, the ratio F/(F+P) is also used to indicate the source of the PAHs and ratios lower than 0.4 would suggest petrogenic sources and higher than 0.4 combustion processes. As can be seen, only the two polluted samples collected in the port in Tromso and this could be linked to diesel combustion of road dust, among others (Tobiszewski and Namieśnik, 2012). Finally, though the ratio Phenanthrene/Anthracene is not included in Figure 1, they also suggest a clear pyrogenic source since the ratios are << 10. Only for the farm mussels we can observe a ratio of 9.8.

PCA analysis of chemical burden in soft tissues of mussels showed that the sites defined as reference sites in the present work are different to the ports defined as chemically impacted sites. In addition, the fact that mussels from the ports were affected by different mixtures of metals and PAHs make it expectable to find different kind of responses in certain biomarkers like in the case of LSC, as it will be discussed. Mussels from the WWTP presented low levels of contaminants and were close to the mussels from the reference sites in the PCA analysis, thus, the different biological status of mussels sampled in the WWTP site are probably related to the presence of other kind of pollutants and/or other ecological factors, as it is discussed below. Size did not seem to be causing important differences regarding accumulation of the analyzed pollutants which could indicate that the few differences found in biological condition of small and large mussels are related to physiological differences among size-classes.

Mussels from the WWTP at Trondheim exhibited a dissimilar gonadal stage in comparison with the others from the same area: they were mostly in advanced gametogenesis. It is expectable to find differences in the physiology of mussels that are near to a WWTP effluent due to increased food availability and/or the effect of pollutants that have been previously described in WWTP discharges (Dumas et al., 2020; Preisner et al., 2021). Mussels from the reference site and the port in Trondheim could have performed a secondary spawning in late summer and at the time of this sampling could be facing the start of winter with immature gametes or it could be the start of a winter maturation (Duinker et al., 2008). The gonadal stages described in mussels from Tromsø, advanced gametogenesis or even mature gametes, could be indicative of an upcoming late autumn spawning process or a more advanced winter maturation process which seems to be a seasonal phenomenon previously reported in the area (Fokina et al., 2018; Storhaug et al., 2019). The factors influencing the differences in gamete developmental stages are to be taken into account as they could interfere heavily with the biomarker responsiveness (Benito et al., 2019; Beyer et al., 2017)

The fact that the only statistically significant differences regarding ADG cell density were detected in small mussels from the harbor could indicate lower coping capabilities against moderate chemical insult and a subsequent reduction of the storage material in small sized mussels (Moukrim et al., 2008). It has been reported that growth

rates are faster in smaller (younger) mussels than in larger (older) ones (Duinker et al., 2008). Thus it is expectable to find less reserve material under the same suboptimum conditions in animals with higher energetic requirements. Thus, size (age) of mussels might have important implications in the biomarker responsiveness and the health status of mussels under chronic chemical insult.

Vv_{LPF} presented high inter-individual variability as it could be expected when comparing mussels subdued to different trophic regimes, tides and chemical insult among other environmental variables. It has been reported that Vv_{LPF} is susceptible of changing depending on oxidative stress (Viarengo et al., 1991), but also when certain environmental conditions like long-term food deprivation in winter in the Baltic Sea followed by a subsequent spring bloom (Benito et al., 2019) affect mussels. Dietary variations have also caused differences in laboratory conditions in previous studies (E. Blanco-Rayón et al., 2019a). Thus, low Vv_{LPF} in mussels from the reference site in Tromsø cannot be directly linked to a better environmental status.

An overall higher amount of neutral lipids in digestive cells (Vv_{NL}) of mussels collected in Tromsø than in Trondheim, which is coherent with previous studies where increased lipid content was described in mussels from artic latitudes (Fokina et al., 2018). An exception is the decreased Vv_{NL} value found in mussels from the harbor in Tromsø, which could be explained as a depletion of lipids under slight chemical stress (Guerlet et al., 2007) and more precisely by mild metal pollution (Zorita et al., 2006). Previous studies in the Bay of Biscay (Garmendia et al., 2010) reported reference Vv_{NL} values of 0.05-0.1 µm3/ µm3 in October which is only comparable with the results obtained in Trondheim in the present work. Mussels from the harbor in Tromsø presented Vv_{NL} values that were higher or similar to the higher part of the Bay of Biscay range while mussels from the reference site were way above the described range. These results are concordant with previous works (Brooks et al., 2015) and they confirm that Norwegian *M. edulis* naturally display higher Vv_{NL} values than the mussels in the Bay of Biscay. However, the lower Vv_{NL} and ADG index levels described in mussels from the harbor in Tromsø may be indicative of some stress source affecting mussels inhabiting this area.

Lysosomal membrane stability test has been widely used as an indicator of general stress in mussels (Benito et al., 2019; E. Blanco-Rayón et al., 2019b; Izagirre and Marigómez, 2009). In the present study LMS displayed the lowest values in mussels from the harbors of Trondheim and Tromsø, while the mussels from the WWTP showed intermediate values between the ones present in the harbor and in the reference sites. Although the measured responses are in concordance with the relative burden of pollutants in the tissues, both the highest and the lowest values are below what it was expectable taking into account the pollutant concentration in tissues. Threshold levels in mussels from the bay of Biscay are stablished in >20 minutes for pristine environmental conditions (Marigómez et al., 2013), but in the present study the reference sites and the WWTP present values that are between 10-20 minutes which correspond to tolerable environmental condition. Mussels sampled in the harbors are, on the other hand, in the range of delicate environmental condition (5-10 min). These results are in concordance with previous studies that compared the response of certain biomarkers in Norwegian *M. edulis* with Basque *M. galloprovincialis* (Brooks et al., 2015), where slightly lower LP were measured in Norwegian mussels compared to Basque mussels. However, it is not possible to apply the thresholds that are stablished for the Bay of Biscay in the present study, as this is the first effort to define the baseline values of certain biomarkers in the sampled latitudes. Nevertheless, it is necessary and useful to apply the threshold values of the *Mytilus sp.* battery of biomarkers that are well stablished in other geographical areas as they give critical information of the responsiveness of these biomarkers in the species.

Maximum values registered in the current study regarding Vv_{LYS} are way above the threshold values described in the Bay of Biscay for bad environmental condition (Marigómez et al., 2013), furthermore, the highest values are displayed by mussels from the reference sites in Trondheim and Tromsø. The implications of these results may be important and we are not able to explain the reason behind with the information of the present research work. In any case, it cannot be discarded the seasonal or geographical effects (Benito et al., 2019) or even the effect of undetected pollutants, although the latter is not probable taking into account the characteristics of the sampling sites. Nevertheless it is remarkable that previous studies (Brooks et al., 2015) reported higher

Vv_{LYS} values in *M. edulis* than in *M. galloprovincialis*, being these comparable to the ones reported in the present study. When comparing lysosomal structural changes between the sampling groups in the present work, no significant differences were detected among the small mussels, as the Vv_{LYS} remained similar due to a high variability in the groups. The lack of responsiveness towards chemical insult that has been described in previous studies (Izagirre and Marigómez, 2009) could have been masked by the effect of a variety of natural confounding factors that caused higher variability in the small mussels (Izagirre et al., 2008). In the case of large mussels, lower Vv_{LYS} values were measured in the Trondheim harbor, which presented smaller lysosomes in higher numbers, being this alteration concordant with the responses to metal pollution in Norwegian *M. edulis* as reported by Brooks et al., 2015 or to the low concentration of organic contaminants (Etxeberria et al., 1994). The lack of differences between both sites in large mussels from Tromsø could be a mixture of mild pollutant levels in the harbor and the confounding effect of the Gymnophallidae like trematodes found in this locality, as it will be discussed below. LSC proved to be a valuable tool to assess general stress responses in wild mussels in the North Atlantic Sea, but in order to link alterations in this endpoint to the effect of pollutants it is necessary to clarify other confounding factors such as parasitism and different threshold levels found naturally under certain seasonal and latitudinal circumstances.

At higher complexity levels of biological organization, the three tissue level biomarkers used in the present work displayed similar trends at least in the clearest responses. Vv_{BAS} was higher in small mussels from the harbors in Trondheim and Tromsø which could be related to chemical stress (E. Blanco-Rayón et al., 2019c). Although Vv_{BAS} values in those groups are remarkably high for the relatively mild pollutant levels in tissue when compared to the Bay of Biscay thresholds (Marigómez et al., 2013), S. Brooks et al. (2011) demonstrated that Norwegian mussels can reach high Vv_{BAS} levels without grave chemical insult. Large mussels only exhibited significant differences when comparing the individuals sampled in the farm and the ones from the WWTP, presenting enhanced Vv_{BAS} values in the latter which could be related to chemical insult and/or higher parasitic burden (discussed below). Small mussels from the harbour in Tromsø show higher Vv_{BAS} values than the large mussels in the same site, this result could be

linked to a slightly worse histopathological and parasitic status that could enhance the stress signal. It could also be linked to a higher metabolic demand in the small mussels (Duinker et al., 2008) that could lead to more marked stress symptoms coherent with the depletion of the reserve material described earlier. The lowest values in the present study were above 0.1 $\mu m^3/ \mu m^3$ which is the good environmental condition threshold in the Biscay Bay (Marigómez et al., 2013). Thus it could be concluded that the Vv_{BAS} baseline values and its responsiveness towards pollution for Norwegian mussels are higher than the ones stablished for mussels from the Bay of Biscay, being this concordant with data reported in previous studies (Brooks et al., 2011).

Atrophy index determination indicated mild levels of digestive tissue degradation in the more affected groups, which in the case of mussels from the harbor in Trondheim were significant when compared to the ones from the reference site, being this concordant with data of pollutant burden in tissues. Mussels from Tromsø displayed relatively high atrophy levels that in the case of the groups from the reference site could be related to geographical and seasonal effects (Benito et al., 2019) and/or parasitic burden (Cuevas et al., 2015). Mussels from the harbor in Tromsø displayed similar atrophy levels to the ones from the reference site, the cause could be an insufficient concentration of pollutants in the harbor to exert a severe response in this biomarker. Lowest values regarding CTD ratio were measured in the small mussels from the reference site in Trondheim. The response displayed by small mussels from the Trondheim harbor could be caused by chemical insult (Múgica et al., 2015) while the increased values in small mussels from Tromsø could be caused by the effect of parasitism (as discussed below), reproductive cycle and in the case of small mussels from the harbor it could be caused by a combination of parasitism and chemical stress. It is concluded that the present atrophy index and CTD ratio data is valuable as a first step towards the establishment of the thresholds indicating good health status and/or general stress conditions in mussels from the studied areas.

Among the remarkable parasitic content mussels from the WWTP presented relatively high prevalence of Renicolidae like trematodes. It has been demonstrated that mussels collected from benthic intertidal beds present higher parasitic burden than the ones collected from ropes, pylons and similar structures (Buck et al., 2005). In

Trondheim the only mussels collected from the benthic intertidal habitat were the ones from the WWTP, while the ones from the reference site and the harbor were collected from pylons, and the ones from the farm were collected from long lines. In addition, the potentially increased organic matter in the WWTP effluent (Preisner et al., 2021) and the subsequent increased amount of prey (Wołowicz et al., 2006) could attract bigger numbers of mussel-eating seabirds that are the final host of Renicolidae trematodes (Stier et al., 2015). Infection of trematodes from the Renicolidae family are known for causing impairments in mussels regarding clearance rate and even growth rate, but mostly when environmental conditions for the hosts are not optimum as they act as background stressors (Stier et al., 2015; Thieltges, 2006). A sampling site that presents an important trematode prevalence like the WWTP in Trondheim must be approached with caution when assessing the stress levels of mussels via biomarkers, as certain responses could be at least partially caused by parasitic burden and not by chemical stressors. Mussels from Tromsø presented trematode infection too, although Renicolidae like parasites were only found in the reference site. Both sites presented Gymnophallidae like organisms that elicited a response from the host that up to the present date has not been described histologically in *Mytilus* species. Similar host reactions towards Gymnophallidae parasites were described by Kinne (1983) in *Cerastoderma edule* where the recently attached *Gymnophallus minutus* metacercariae is surrounded by columnar epithelium from the pallial line and when the parasite gets totally enclosed by the epithelium the lysis starts. As in the present work is observed cellular debris and the presence of haemocytes in the surrounding tissues is described. Furthermore, mussels infected with the Gymnophallidae like trematode presented different combinations of additional lesions in the mantle that ranged from haemocytic infiltrations in the connective tissue and/or gonad follicles, atresia and brown cell infiltrations in male follicles (lesser extent). Although the host response is similar in both bivalves, the infection intensity is higher in cockles (Goater, 1993) than what is found in mussels in the present study. Taking into account the potential stress source that the histopathologically less worrying Renicolidae like trematodes suppose, the pathogenicity of the Gymnophallidae like parasites in the present study seems to be of critical importance. The stress caused by the immunological response (tissue encapsulation, inflammatory responses) could explain the stress levels in certain

biomarker responses in mussels from the allegedly non-impacted site in Tromsø including lysosomal alterations and high atrophy and CTD levels. Further research is being performed in order to identify the Gymnophallidae like trematode found in the present work with the combination of histopathological and molecular approaches.

Regarding pathological status in mussels sampled in Trondheim the most remarkable results are the higher prevalence of haemocytic infiltrations in small mussels form the harbor and both groups of mussels in the WWTP. It is known that these inflammatory responses are related to starvation, reproductive stress, shell damage, parasitism and exposure to hydrocarbons and metals (Benito et al., 2019; Garmendia et al., 2011). Exposure to chemical stressors is the most probable cause of the haemocytic infiltrations found in mussels from the Trondheim harbor, while mussels from the WWTP might show a high prevalence of inflammatory responses as an outcome of the combination of mild chemical insult and parasitism. In the case of mussels from Tromsø the increased prevalence of haemocytic infiltration could be related to the presence of trematodes as discussed before. The presence of brown cells in gonad follicles and atresia of oocytes could be indicators of ongoing autolysis and resorption processes during gametogenesis, and can be induced when environmental conditions become unfavorable for spawning after gamete maturation (Katarzyna Smolarz et al., 2017; Suárez et al., 2005). Thus it is expectable to find these alterations more commonly in individuals with a more advanced gametogenic state like mussels from the WWTP in Trondheim and mussels sampled in Tromsø. Although the presence of atresia is not necessarily pathological, it seems that the presence of the Gymnophallidae like trematodes might be one of the causes behind the increase of atresia prevalence in mussels from Tromsø. Granulocytomas are inflammatory responses resulting in vascular occlusions (Lowe and Moore, 1979) that have been linked to chronic pollution and the presence of metacercarian parasites (Benito et al., 2019; Garmendia et al., 2011). The latter seems to be the differential reason to explain the relatively high granulocytoma prevalence in Tromsø as the higher pollutant concentration in the harbor did not seem to increase the appearance of these lesions. This reinforces the idea that the Gymnophallidae like parasite is a variable to take into account as a confounding factor to assess the environmental health status by biomarker responses and histopathology.

CONCLUSIONS

The present study is the first step towards the establishment of baseline level values of cell and tissue-level biomarkers and their responsiveness to pollutants in two localities from the Norwegian Sea in subarctic and Arctic latitudes. Although the battery of biomarkers used here proved to be useful to discriminate impacted and non-impacted mussel populations, some confounding factors altering the biological responses have been identified. Geographical/latitudinal factors seemed to be critical regarding the reproductive cycle, reserve material storage and the prevalence of parasites such as Gymnophallidae like trematodes. Size (age) of mussels seemed to be important regarding the physiological condition and the coping capabilities of the individuals towards moderate chemical insult, although this was only detected in some biomarkers in small mussels from the port in Tromsø. On the contrary, the mussels from the reference site in Tromsø displayed general stress responses at different levels, which could be influenced by the pathogenic effect of the Gymnophallidae like trematode and by a more advanced gametogenic developmental stages compared to the mussels from Trondheim, which could lead to misinterpretation of the stress levels in those mussels. All in all, the current work serves as an anchor point both as a reference of the baseline level values of the analyzed endpoints in the studied geographical area and time of the year, and as an indication of the potential extent of the environmental confounding factors causing stress on the analyzed mussel populations.

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Chapter 3: Variability and distribution of parasites, pathologies and their effect on wild mussel (*Mytilus sp*.) populations in different environments along a wide latitudinal span in the Northern Atlantic and Arctic Oceans

Article: Variability and distribution of parasites, pathologies and their effect on wild mussel (*Mytilus sp*) populations in different environments along a wide latitudinal span in the Northern Atlantic and Arctic Oceans. Denis Benito, Dragana Paleček, Xabier Lekube, Urtzi Izagirre, Ionan Marigómez, Beñat Zaldibar, Manu Soto. Marine Environmental Research (Accepted)

ABSTRACT

Histopathological examination in mussels can provide useful information for the diagnosis of ecosystem health status. However, the distribution of parasites in mussels can be conditioned by several environmental factors, including mussel collecting sites or the presence/absence of other species necessary to complete the complex life cycle of certain parasites. Thus, these variables could not only govern the parasitic burden of mussels but also the presence of pathologies associated with parasitism . The aim of this study is to identify the histopathological alterations which could be indicative of a health status distress along a wide latitudinal span in the Northern Atlantic and Arctic Oceans in mussels of two size-classes sampled in clean and impacted sites. A latitudinal gradient is clearly observed in gamete developmental stages as northern and southern mussels presented different conditions at the same period. Furthermore, the size/age relationship seemed not to be comparable latitudinally with evident differences in the reproductive cycle and the appearance of related pathologies. In addition, specific parasitic profiles ruled by latitudinal conditions and the settlement of mussels in the shore (horizontal/vertical) have been demonstrated to be of a great importance in the health condition of mussels. Furthermore, the present work provides the first histological description of Gymnophallidae-like parasites causing an important pathogenic effect in Tromsø and Iceland plus the report of possible high mortality events related to a high prevalence of granulocytomas in Scotland and Germany.

LABURPENA

Muskuiluen azterketa histopatologikoak informazio baliagarria eman dezake ekosistemen osasun egoeraren diagnostikoa egiteko. Hala ere, muskuiluetan parasitoen banaketa hainbat ingurumen faktorek baldintzatu dezakete, besteak beste, muskuiluak biltzeko guneak edo parasito batzuen bizi ziklo konplexua osatzeko beharrezkoak diren beste espezie batzuen presentzia/absentzia. Hortaz, aldagai hauek muskuiluen parasitoen edukia ez ezik, parasitismoari lotutako patologiak ere arau ditzakete. Ikerketa honen helburua Ipar Ozeano Atlantiko eta Ozeano Artikoetan leku ez kutsatu eta kutsatuetan lagindutako bi tamainetako muskuiluen osasun egoera kaltetuaren adierazgarri izan daitezkeen alterazio histopatologikoak identifikatzea da. Gradiente latitudinal argia ikusi zen gametoen garapen faseetan, iparraldeko eta hegoaldeko muskuiluek aldi berean baldintza desberdinak aurkeztu baitzituzten. Gainera, tamaina/adin erlazioa ez zela latitudinalki konparagarria zirudien, ugaltze zikloan eta erlazionatutako patologien agerpenean desberdintasun nabariak sortu zuena. Hare eta gehiago, faktore latitudinalek eta muskuilen finkatze tokiak (horizontal/bertikal) araututako profil parasitiko espezifikoak sortu zituzten, garrantzi handia izan zutenak muskuiluen osasun egoeran. Gainera, lan honek Tromsø-n eta Islandia-n efektu patogeno garrantzitsua eragiten duen Gymnophallidae antzeko bizkarroien lehen deskribapen histologikoa eskaintzen du eta Eskozian eta Alemanian granulozitomen prebalentzia altuarekin lotutako hilkortasun handiko gertakarien berri ematen du.

INTRODUCTION

Mussels (*Mytilus sp.*) are widely used sentinel organisms in pollution monitoring programs to determine the health status of coastal ecosystems and the use of the biomarker approach has been widely established (Brenner et al., 2014; Nasci et al., 2002). Even though this approach has been proved to be useful, the interpretation of biological responses related to chemical insult can be biased due to the interactions between pollutants and natural factors or between natural and physiological factors. Consequently, these possible confounding factors must be precisely identified (Benito et al., 2019; Beyer et al., 2017; Fokina et al., 2018; Leiniö and Lehtonen, 2005; Nahrgang et al., 2013). In addition, the definition of the range of natural variability and how it may influence the correct interpretation of the biological effects caused by pollution is of major importance (Beyer et al., 2017; Izagirre et al., 2008)..

Histopathological examination in mussels can provide useful information for the diagnosis of ecosystem health status (Garmendia et al., 2011). Changes in the parasitic burden and the development of inflammatory and degenerative lesions, which are among the most common histopathological abnormalities, can be caused by disturbances at low levels of biological complexity and may pose deleterious consequences for the health status of populations (Moore and Simpson, 1992). Even though histopathology in mussels has been previously used for the assessment of the effects of exposure to PAHs, PCBs and metals (Auffret, 1988; Lowe and Pipe, 1987; Marigómez et al., 2006), care must be taken when directly using histopathology as a biomarker of exposure since it demonstrated to be dependent on parasite infestation of wild mussel populations (Bignell et al., 2011, 2008; Cuevas et al., 2015; Villalba et al., 1997).

The distribution of parasites in mussels can be conditioned by several environmental factors, including mussels collecting sites (Buck et al., 2005) or the presence/absence of other species necessary to complete the complex life cycle of certain parasites (Goater, 1993). Thus, these variables could not only govern the parasitic burden of mussels but also the presence of pathologies associated with parasitism. In addition, the reproductive cycle of mussels is another important

confounding factor that is known to influence biomarker responsiveness (Benito et al., 2019), which at the same time is known to be influenced by seasonality (Sheehan and Power, 1999) and age of mussels (Handå et al., 2011).

The aim of this study is to identify the histopathological alterations that could be indicative of a health status distress that might potentially cause an altered biological response influencing the biomarker responsiveness to pollutants on mussels from the Northern Atlantic Ocean and Arctic Ocean along a wide latitudinal span. For this purpose, a histopathological assessment was carried out analyzing the gamete developmental stages, the presence of parasitism and the appearance of pathologies in the mantle and digestive gland of mussels. Furthermore, the possible relationships between the presence of parasites and the incidence of pathologies were established and the most influential environmental factors on the prevalence of the histopathological abnormalities were identified. The potential influencing factors taken into account in the present study were anthropogenic impact, reproductive cycle, mussel size as a proxy of age, collection site characteristics and geographical causes. The possible pathogenicity of the histopathological alterations and its potential effect on biomarker responsiveness are also discussed considering the implications these variables might have on classic biomonitoring design guidelines.

MATERIAL AND METHODS

Sampling strategy

Mussel collection was carried out in late summer 2017 at different locations of the northern Atlantic Ocean and Arctic Ocean along a wide latitudinal span (Fig.1).

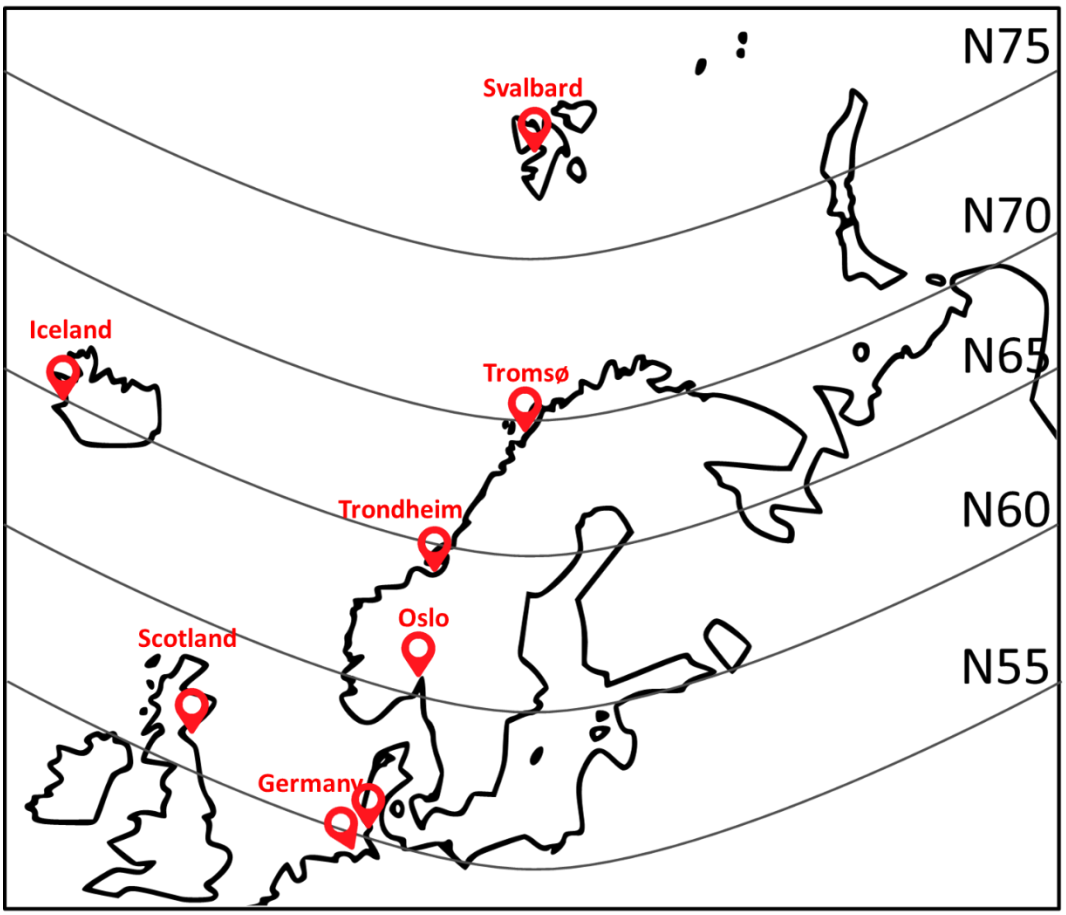

Figure 1: Map indicating the geographical location of the sampling sites

The sampling sites where mussels were collected are described in Table 1, including GPS coordinates, date of collection, type of habitat, mussel settlement in the spot (vertical versus horizontal) and their classification according to latitudinal categories. Latitudinal categories were defined as Northernmost locations (sampling sites above the arctic circle), Intermediate locations (sampling sites located between the arctic circle and the 60° N parallel) and Southernmost locations (sampling sites located below the 60° N parallel).

In order to compare sampling sites depending on their anthropogenic impact (non-impacted versus impacted), sites were selected following the advice of local researchers that use mussels from these sites in biomonitoring programs or with research purposes. Thus, the non-impacted sites (in green, Table 1) are relatively low populated open (high water renewal rate) natural environments. On the other hand, impacted sites (in yellow in the Table 1) are more heterogeneous: Eckwarderhörne is located in the mouth of an enclosed bay located in the vicinity of Wilhelmshaven and Bremerhaven which are relatively highly populated cities with important shipbuilding and commercial port activities. Similarly, Leith is in the harbor area of Edinburgh which may also be impacted by other human activities such as agriculture, oil industry and a nuclear plant. The sampling site of Malmøya is an enclosed marina in Oslo while WWTP in Trondheim, corresponded to a rocky beach close to a wastewater treatment plant effluent. The rest of the impacted sampling sites in Trondheim, Tromsø and Reykjavik were located in enclosed ports.

Mussels of two different sizes (small: 2-3 cm and large: 3.5-4.5 cm) were sampled, transported to the laboratory in air at ambient temperature and dissected immediately except in the two German sampling points where they were dissected *in situ*. All the collected mussels were intertidal, picked up from the first metre of the intertidal zone except in Malmøya (Oslo) and Svalbard where the mussels were subtidal and retrieved from a floating dock and underwater by scuba diving, respectively.

Table 1: Precise information of the sampling sites including anthropogenic impact, geographical location, date of sampling, tidal regime, characteristics of sampling sites, distribution of mussels and the latitudinal clusters in which they were classified. Green: non-impacted, yellow: presence of important anthropogenic impact. H: Horizontal, V: Vertical, S: Southernmost, I: Intermediate, N: Northernmost

Country	Location	Latitude	Longitude	Date	Tidal regime	Sampling site type	Settlement of mussels	Latitudinal cluster
Germany	Königshafen	55.042374	8.449102	10/10/2017	Intertidal	Mussel bed	H	S
	Eckwarderhörne	53.520301	8.231816	11/10/2017	Intertidal	Rock breakwater	H	S
Scotland	St. Andrews	56.333602	-2.776173	21/09/2017	Intertidal	Rocky beach	H	S
	Leith	55.977359	-3.140404	20/09/2017	Intertidal	Rocky beach	H	S
Iceland	Havfjördur	64.358154	-21.486458	07/09/2017	Intertidal	Rocky beach	H	I
	Reykjavik (port)	64.155605	-21.939218	08/09/2017	Intertidal	Rock wall	V	I
Norway	Oslo (Malmøya)	59.873896	10.757743	15/09/2017	Subtidal	Floating jetty	V	S
	Oslo (Drøbak)	59.615889	10.652007	18/09/2017	Intertidal	Rocky shore	H	S
	Trondheim (port)	63.442692	10.425494	20/09/2017	Intertidal	Pylons	V	I
	Trondheim (WWTP)	63.444867	10.341331	21/09/2017	Intertidal	Rocky beach	H	I
	Trondheim (Rissa)	63.561753	9.899776	21/09/2017	Intertidal	Pylons	V	I
	Tromsø (port)	69.654177	18.968459	18/09/2017	Intertidal	Concrete surface	H	N
	Tromsø (aquarium)	69.642089	18.94639	18/09/2017	Intertidal	Rocky beach	H	N
	Svalbard (Longyearbyen)	78.236081	15.606482	21/09/2017	Subtidal	Pylons	V	N

Histological sample processing and gamete developmental stages

Histological sample processing and gamete developmental stages were performed as described in chapter 1.

Histopathological analysis

Slides of 20 mussels per sampling campaign were examined individually under the light microscope using 10×, 20× or 40× objective lenses. Quantitative scores were made by keeping a running count of the incidences as the slide was scanned to avoid re-examination of each slide multiple times for each category. The prevalence of rickettsia/chlamydia like organisms (RLO/RCO), MPX intracellular ciliates, *Mytilicola sp.* copepods, trematode sporocysts (Bucephalidae) and trematode metacercariae

(Gymnophallidae and Renicolidae like trematodes) were analysed as indicated by Kim et al. (2006). In addition, the prevalence of certain tissue conditions was analyzed, including cases of brown cell (ceroid bodies or pigment cells), aggregates in digestive gland and gonad, follicular atresia, granulocytomas and haemocytic infiltration in digestive gland and gonad, without distinction between focal and diffuse. The intensities of RLO/CLO, MPX, Gymnophallidae and Renicolidae like trematodes and granulocytoma were calculated quantitatively. The obtained scores were used to compute the following parameters: Prevalence = N_H/N_S, and Intensity = S_P/N_H; where N_H is the number of specimens hosting parasites or pathologies, N_S is the number of specimens analyzed per sample, S_P is the score corresponding to each parasite and pathology recorded. Prevalence and intensity provide information about the incidence of each parasite and tissue condition.

The intensities of trematode sporocyst (Bucephalidae) infection and atresia were assessed as described by Kim et al. (2006).

Statistical analysis

Statistical analysis was carried out with the aid of the SPSS/PC+ statistical package V.24 (SPSS Inc., Microsoft Co.). Pearson's test ($P<0.01$, $P<0.05$) was used for the correlation of the prevalence of different parasites and pathologies. Kruskal-Wallis test was used to compare the distribution ($P<0.05$) of the prevalence of parasites and pathologies when sampling groups were clustered depending on sampling sites with or without high anthropogenic activity, characteristics of the substrate in which mussels were sampled, size of the mussels and latitudinal categories.

RESULTS

Gamete developmental stages

In general terms, mussels from all sites showed signs of an ongoing spawning process or a recent spawning event (Fig. 2). However, it should be noted that in southern sites higher prevalence of post-spawned mussels is observed while in the northern sites prevalence of spawning (Fig. 3A, 3B) mussels is higher. On the other hand, certain differences can also be appreciated when comparing mussel sizes, as the sum of the percentages of mussels spawning and post-spawning in small mussels was around 50% or below with the exception of mussels from the WWTP in Trondheim, Tromsø and Svalbard. Conversely, large mussels presented a sum of percentages of spawning and post-spawning stages close to 80% or above, with the exception of Icelandic mussels exhibiting values around 50-60%.

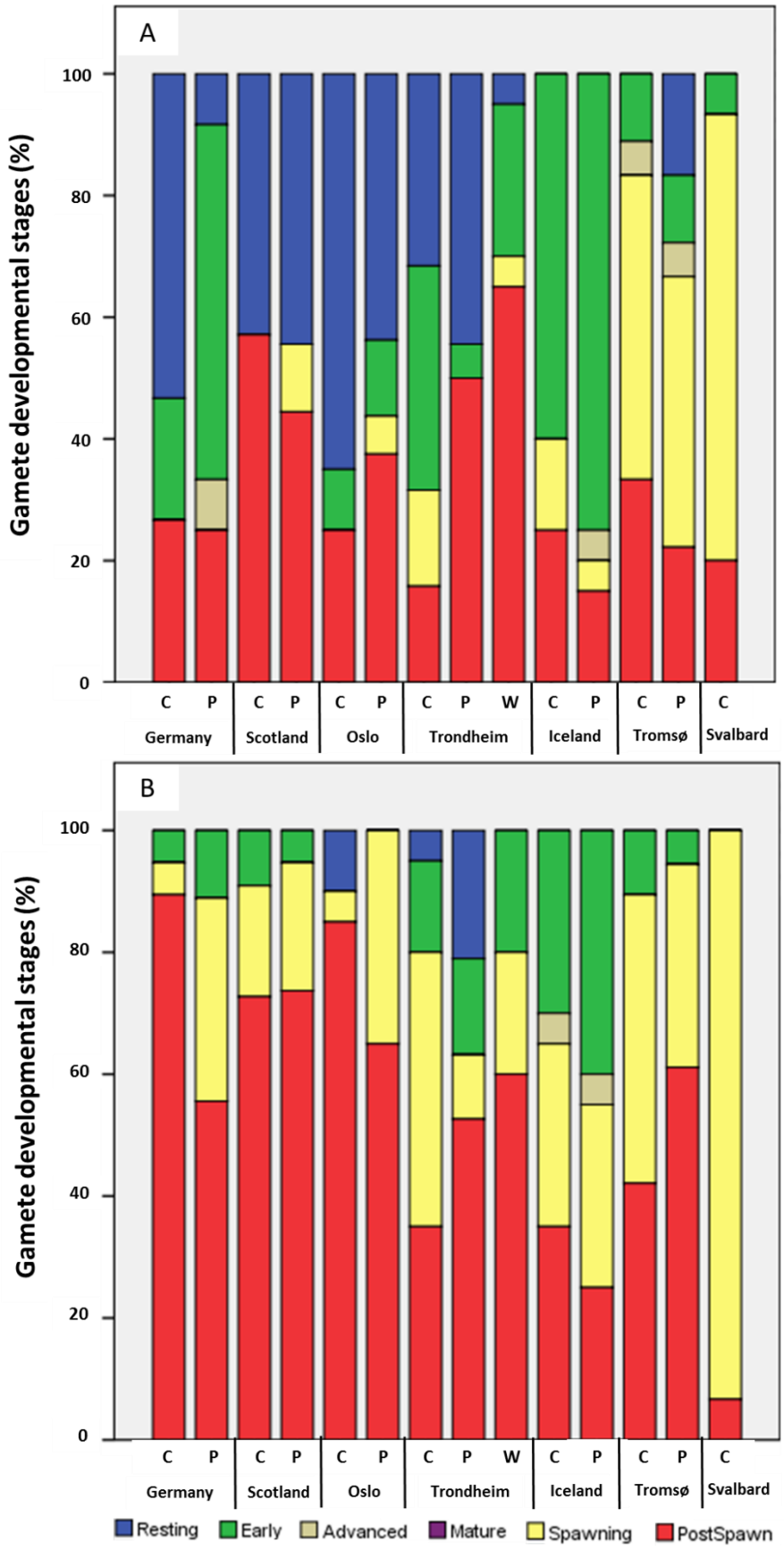

Figure 2: Graph represents percentages of gamete developmental stages in small mussels (A) and in large mussels (B). Letters indicate anthropogenic impact: C: Clean, P: Presence of Anthropogenic activities.

Parasite and histopathological alterations: prevalence and intensities

Regarding parasite infestation the prevalence of microorganisms (Table 2) such RLO/CLO in the digestive gland of mussels (Fig. 4A) was low overall, with the highest prevalence (%20) being recorded in small mussels from the clean site in Oslo. Overall, MPX (Fig. 4B) prevalence was below 20% except in large mussels from the harbor in Trondheim where more than 50% of the individuals exhibited these intracellular ciliates. The prevalence of *Mytilicola sp.* (Fig. 4D) was high in mussels sampled in Germany (33%-66.7%), but the prevalence was low (<15%) in the rest of the groups that presented this copepod. On the contrary, trematode sporocyst infection (Bucephalidae) (Fig. 4C) was only found in 3 groups and their prevalence was also very low (<11%). Gymnophallidae-like parasites (Fig. 4E, 4G) were only found in Tromsø and Iceland, and except in large mussels from the clean site in Tromsø where the prevalence was 31.58%, the rest of the groups presented lower percentages (<16%). The prevalence of Renicolidae-like trematodes (Fig.4F) in those groups ranged between 35-76.47 % except in the clean site of Tromsø where it reached only to the 5.26 %.

Table 2: Prevalence (%) of RLO/CLO, MPX intracellular ciliates, Mytilicola sp., Bucephalidae trematode sporocysts, Gymnophalidae and Renicolidae trematodes, brown cell infiltration in gonad, atresia of the oocytes, haemocytic infiltration in gonad, brown cell infiltration in digestive gland, granulocytoma and haemocytic infiltration in digestive gland. DG: Digestive gland, G: Gonad.

Location	Impact	Size	RLO/ CLO	MPX	Mytilicola sp.	Bucephalid.	Gymnophallid.	Renicolid.	Brown Cell Inf.(G)	Atresia	Haem. Inf. (G)	Brown Cell Inf. (DG)	Granulocytoma	Haem. Inf. (DG)
Germany	Clean	Small	0	0	52.94	0	0	76.47	5.88	0	0	0	11.76	23.53
		Large	0	10	60	0	0	65	20	0	15	70	50	65
	Impacted	Small	0	0	66.67	0	0	16.67	0	0	0	0	0	16.67
		Large	0	0	33.33	0	0	38.89	33.33	9.09	5.56	61.11	16.67	66.67
Scotland	Clean	Small	0	21.43	0	0	0	0	0	0	14,29	0	0	64.29
		Large	0	14.29	0	7.14	0	21.43	7.14	0	28.57	35.71	42.86	71.43
	Impacted	Small	0	11.11	5.56	0	0	66.67	0	0	11.11	5.56	0	66.67
		Large	5.26	0	5.26	0	0	42.11	5.26	0	47.37	15.79	47.37	78.95
Oslo	Clean	Small	20	10	0	0	0	0	0	0	0	25	0	30
		Large	0	0	0	0	0	0	10	5.56	35	60	0	20
	Impacted	Small	0	0	0	0	0	0	0	0	20	25	0	65
		Large	5	5	0	0	0	0	5	7.14	50	50	10	45
Trondheim	Clean	Small	0	0	0	0	0	0	0	0	0	0	0	25
		Large	0	15	0	0	0	0	0	41.67	0	0	0	20
	Impacted	Small	0	15.79	0	0	0	0	0	0	0	0	0	0
		Large	0	52.63	0	0	0	0	0	0	0	0	0	5.26
	WWTP	Small	0	0	5	0	0	35	10	14.29	5	5	0	65
		Large	0	15	10	0	0	35	10	8.33	5	0	0	65
Iceland	Clean	Small	5	0	15	0	5	70	0	0	20	0	0	15
		Large	5	0	0	0	10	50	5	11.11	50	10.53	0	30
	Impacted	Small	0	0	0	0	0	0	5	0	5	0	0	65
		Large	0	0	0	0	5	0	0	25	5	0	0	25
Tromsø	Clean	Small	5.26	0	5.26	10.53	15.79	5.26	0	50	5.26	5,26	5.26	78.95
		Large	0	0	5.26	0	31.58	5.26	0	54.55	47.37	0	5.26	52.63
	Impacted	Small	5.56	0	0	5.56	0	0	0	11.11	0	0	0	22.22
		Large	5.56	0	0	0	5.56	0	16.67	30	5.56	0	0	55.56
Svalbard	Clean	Small	0	0	0	0	0	0	0	88.38	13.33	0	0	6.67
		Large	0	0	0	0	0	0	6.67	100	6.67	0	0	13.33

Overall, RLO/CLO infection intensity was low, except in large mussels from the impacted site of Oslo where up to 134 organisms were counted in a single slide. All the MPX ciliates counts were below 50 specimens per slide. Trematode sporocyst (Bucephalidae) infection intensity was high in large mussels from the clean site in

Scotland and small mussels from the clean site in Tromsø, and intermediate in small mussels from the impacted site in Tromsø. Only one specimen of Gymnophallidae-like trematode was found in most of the infected mussels except in the large mussels sampled in the clean site in Iceland and in the impacted site in Tromsø whose mean infection intensity was of 2 specimens per mussel section. Renicolidae-like trematode mean infection intensity ranged from 1 to 5 specimens per slide.

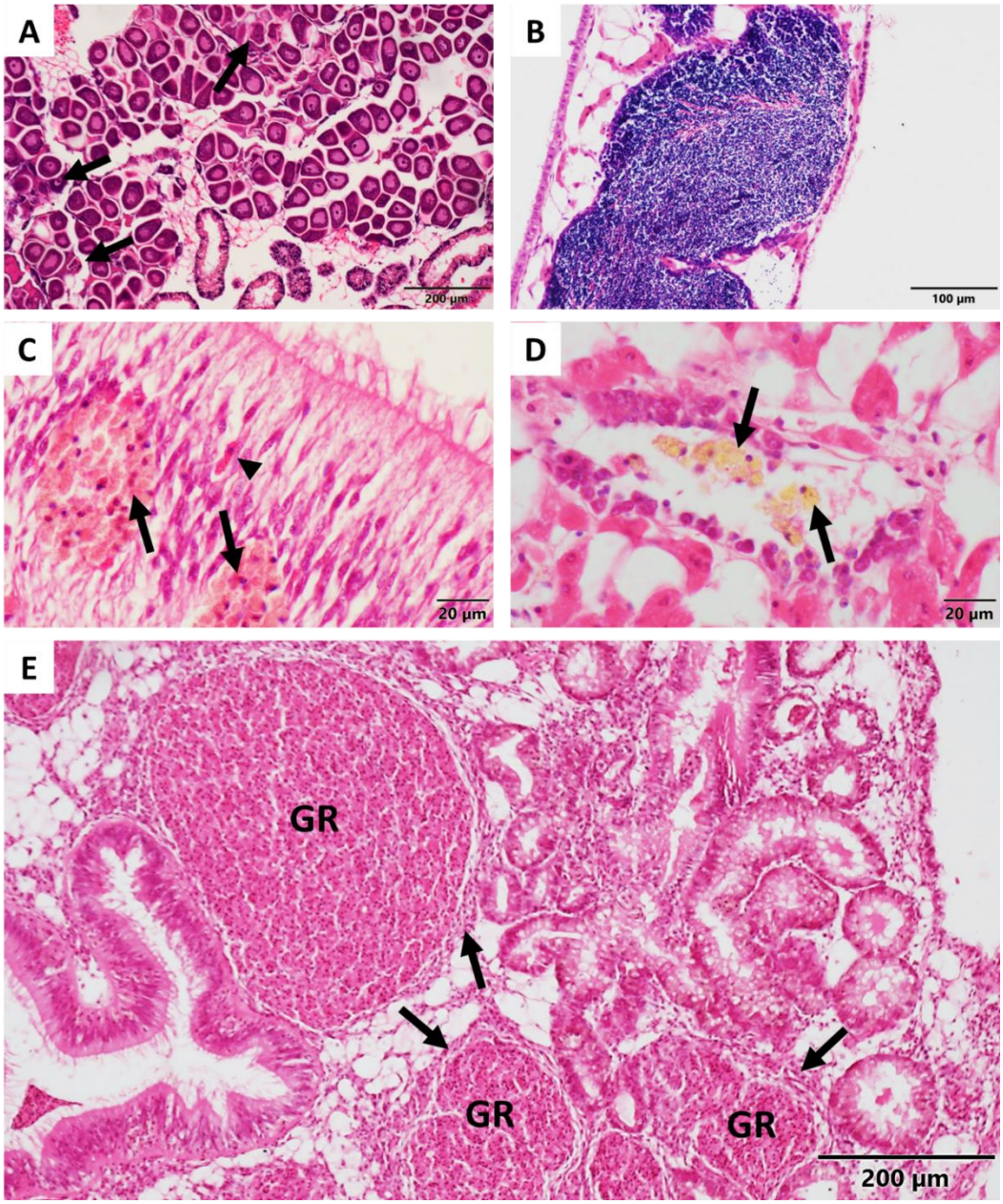

Figure 3: Micrographs (Hematoxylin-Eosin staining) showing A: Spawning process of a female gonad and atresic oocytes (arrows) in a mussel from Svalbard. B: Spawning process of a male gonad in a mussel from Svalbard. C: Brown cell infiltration (arrows) and a haemocyte (arrowhead) in diapedesis in the digestive epithelium in a mussel from Germany. D: Brown Cell infiltration in gonad follicles (arrows) of a mussel from Germany. E: Multiple granulocytomas consisting of a mass of accumulated granulocytes (GR) surrounded by layers of flattened, epithelioid cells (arrows).

Regarding the mantle pathologies, brown cell infiltration (Fig. 3D) was found in a relatively small number of individuals. The highest prevalence (33.33%) was detected in large mussels from the impacted site in Germany while for the rest of the groups it was below 20%. Atresia (Fig. 3A) was very prevalent in both small and large mussels from Svalbard (88.38% and 100% respectively) followed by mussels from the clean site in Tromsø (50% in small mussels and 54.55% in large mussels). Atresia levels were below 42% for the rest of the groups.

Highest prevalences of haemocytic infiltration in gonad were seen in large mussels from the impacted site in Oslo and in the clean site in Iceland (both 50%), the impacted site in Scotland and the clean sites in Tromsø (47.37%), the clean site in Oslo (35%) and finally Scotland (28.57%). The rest of the groups displayed a maximum prevalence of 20%.

As far as the brown cell infiltration in the digestive gland (Fig. 3C) is concerned, the prevalence was low (<11%) in small mussels from Scotland, small mussels from the WWTP in Trondheim, small mussels from the clean site in Tromsø and large mussels from the clean site in Iceland, while in the rest of the groups it was much higher with values ranging from 15% to 70%.

When it comes to granulocytoma prevalences, (Fig. 3E) the highest were found in large mussels from the impacted site in Germany (50%) and large mussels from the impacted and clean sites in Scotland (47.37%, 42.86% respectively). The rest of the groups in which granulocytomas were detected displayed a prevalence of less than 17%.

The highest haemocytic infiltration (Fig. 3C) prevalence in digestive gland and gills was found in small mussels from the impacted site in Oslo, large mussels from Germany, the four groups from Scotland, both groups from the WWTP in Trondheim, small mussels from the clean site in Tromsø and small mussels from the impacted site in Iceland, all of them ranging from 64.29% to 78.95%. Mussels from the clean site in Oslo, large mussels from the impacted site in Oslo, small mussels from Germany, mussels from the clean site in Trondheim, all groups from Tromsø except small mussels from the clean site and both groups of large mussels from Iceland displayed prevalence ranging

between 20% and 55.56%. The rest of the groups displayed a haemocytic infiltration prevalence below 16.67%.

Mean atresia intensity was high in large mussels from the impacted site in Oslo (4), the impacted site in Germany (3), the clean site in Trondheim (3), large mussels from Svalbard (3.5) and small mussels from Svalbard (3.38). Mean atresia intensity was intermediate in large mussels from the WWTP in Trondheim (2), small (2.62) and large (2.42) mussels from the clean site in Tromsø, large mussels from the impacted site in Tromsø (2.17), large mussels from the clean (2.5) and impacted (2.83) sites in Iceland. The rest of the groups that displayed atresia presented low intensity (1). Large mussels from the clean site in Scotland presented more than 10 granulocytomas per slide in several individuals. Large mussels from the clean and impacted sites in Germany and from the impacted site in Scotland presented multiple granulocytomas (1-10) per slide, while mussels from the rest of the groups presenting this pathology displayed a single granulocytoma per slide.

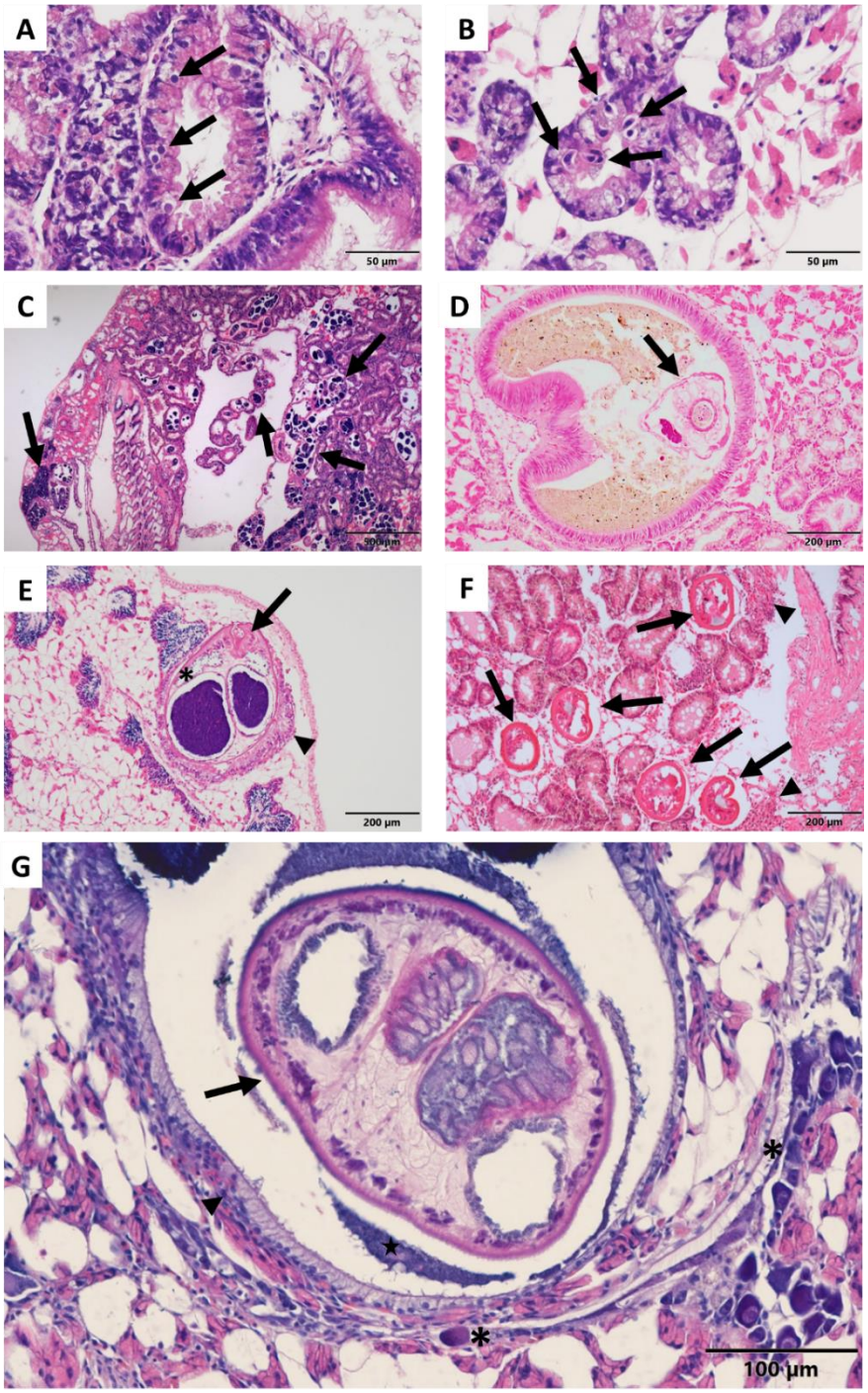

Figure 4: Micrographs (Hematoxylin-Eosin staining) showing A: RLO/CLO parasites (arrows) in digestive cells of mussel from Oslo. B: MPX intracellular ciliates (arrows) in digestive cells of a mussel from Oslo. C: Bucephalidae trematode sporocysts (arrows) infecting mantle, kidney and digestive gland tissue in a mussel from Scotland. D: *Mytilicola sp*. (arrow) in the digestive tract of a mussel from Germany. Gymnophallidae trematode metacercariae (arrow), columnar epithelium of host mantle tissue (arrowhead) and mechanical disruption of the gonad follicle (asterisk) of a mussel from Iceland. F: Multiple Renicolidae trematode metacercariae (arrows) infecting digestive gland tissue and immune responses of the host as focal haemocytic infiltration (arrowheads) in a mussel from Germany. G: Detailed view of a Gymnophallidae trematode metacercariae (arrow), columnar epithelium of host mantle tissue (arrowhead), cellular debris (star) and mechanical disruption of the gonad follicle (asterisks) of a mussel from Tromsø.

Correlation analysis between the prevalence of parasites and histopathological alterations

Pearson's correlation (Table 3) showed significant correlations between the prevalence of *Mytilicola sp.* and Renicolidae-like trematodes. The prevalence of Gymnophallidae-like trematodes was positively correlated with haemocytic infiltration in gonad while the prevalence of Renicolidae like trematodes was also positively correlated to the granulocytoma prevalence in the digestive gland and mantle. The presence of brown cell infiltration in the digestive gland was correlated with the presence of brown cell infiltrations in gonad and granulocytoma prevalence while its prevalence was correlated with the occurrence of granulocytoma and hemocytic infiltration. The presence of granulocytomas was positively correlated to haemocytic infiltration in digestive gland.

Table 3: Pearson's correlation for the prevalence of all the pathologies and parasites found. ** in green: Correlation is significant at the 0.01 level (2-tailed). * In yellow: Correlation is significant at the 0.05 level (2-tailed). DG: Digestive gland, G: Gonad.

		Bucephalidae	MPX	Mytilicola sp.	Gymnophallidae	Renicolidae	Brown Cell Inf. (DG)	Brown Cell Inf. (G)	Atresia	Granulocytoma	Haem. Inf. (G)	Haem. Inf. (DG)
RLO/CLO	Correlation	0.137	-0.093	-0.186	0.054	-0.073	0.067	-0.128	-0.113	-0.033	0.057	-0.010
	Sig. (2-tailed)	0.489	0.638	0.344	0.785	0.710	0.733	0.516	0.568	0.867	0.775	0.960
	N	28	28	28	28	28	28	28	28	28	28	28
Bucephalidae	Pearson Correlation		-0.051	-0.125	0.228	-0.128	0.003	-0.123	0.114	0.203	-0.059	0.291
	Sig. (2-tailed)		0.798	0.527	0.243	0.516	0.988	0.534	0.565	0.301	0.766	0.134
	N		28	28	28	28	28	28	28	28	28	28
MPX	Pearson Correlation			-0.136	-0.215	-0.124	-0.071	-0.158	-0.240	0.003	-0.211	-0.152
	Sig. (2-tailed)			0.490	0.273	0.531	0.720	0.422	0.218	0.989	0.281	0.441
	N			28	28	28	28	28	28	28	28	28
Mytilicola sp.	Pearson Correlation				-0.104	.561**	0.259	0.369	-0.233	0.353	-0.192	0.017
	Sig. (2-tailed)				0.599	0.002	0.184	0.054	0.232	0.065	0.328	0.930
	N				28	28	28	28	28	28	28	28
Gymnophallidae	Pearson Correlation					-0.058	-0.194	-0.157	0349	-0.090	.390*	0.151
	Sig. (2-tailed)					0.771	0.323	0.426	0.069	0.648	0.040	0.442
	N					28	28	28	28	28	28	28
Renicolidae	Pearson Correlation						0.162	0.316	-0.329	.403*	0.135	0.232
	Sig. (2-tailed)						0.409	0.101	0.087	0.033	0.492	0.234
	N						28	28	28	28	28	28
Brown Cell Inf. (DG)	Pearson Correlation							.632**	-0.275	.556**	0.371	0.296
	Sig. (2-tailed)							0.000	0.157	0.002	0.052	0.127
	N							28	28	28	28	28
Brown Cell Inf. (G)	Pearson Correlation								-0.087	.416*	0.021	.379*
	Sig. (2-tailed)								0.661	0.028	0.914	0.046
	N								28	28	28	28
Atresia	Pearson Correlation									-0.218	-0.003	-0.181
	Sig. (2-tailed)									0.265	0.987	0.356
	N									28	28	28
Granulocytoma	Pearson Correlation										0.359	.471*
	Sig. (2-tailed)										0.060	0.011
	N										28	28
Haem. Inf. (G)	Pearson Correlation											0.259
	Sig. (2-tailed)											0.184
	N											28

Factors affecting parasites and pathology prevalences

Clean vs. impacted sites: No significant differences were found between mussels sampled in clean and impacted sites when comparing the prevalence of all parasites and pathologies (results not shown).

Vertical vs. horizontal settlements: Significant differences were found between mussels sampled from horizontal and vertical substrates when comparing the prevalence of parasites and pathologies. The prevalences of *Mytilicola sp.* (Fig. 5A), Renicolidae-like trematode (Fig. 5B) and haemocytic infiltration (Fig. 5C) in the digestive gland and the gills were more common in horizontal substrates than in vertical ones.

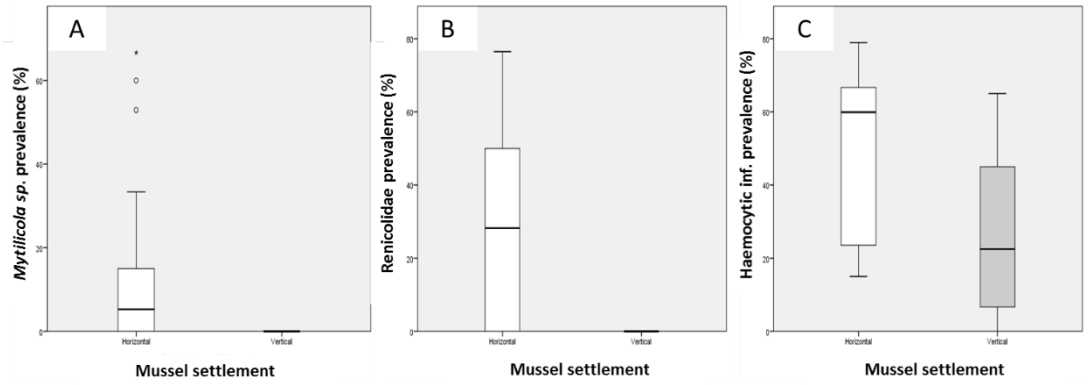

Figure 5: Changes in distribution of the prevalence (%) of *Mytilicola sp*. (A), Renicolidae-like trematodes (B) and haemocytic infiltration (C) in digestive gland of mussels. Differences are statistically significant (p<0.05). DG: Digestive Gland.

Size (age) effect: Large sizes were significantly related with brown cell (Fig. 6A) and haemocytic infiltrations (Fig. 6B) in gonad while the rest of pathologies were not related with mussel size.

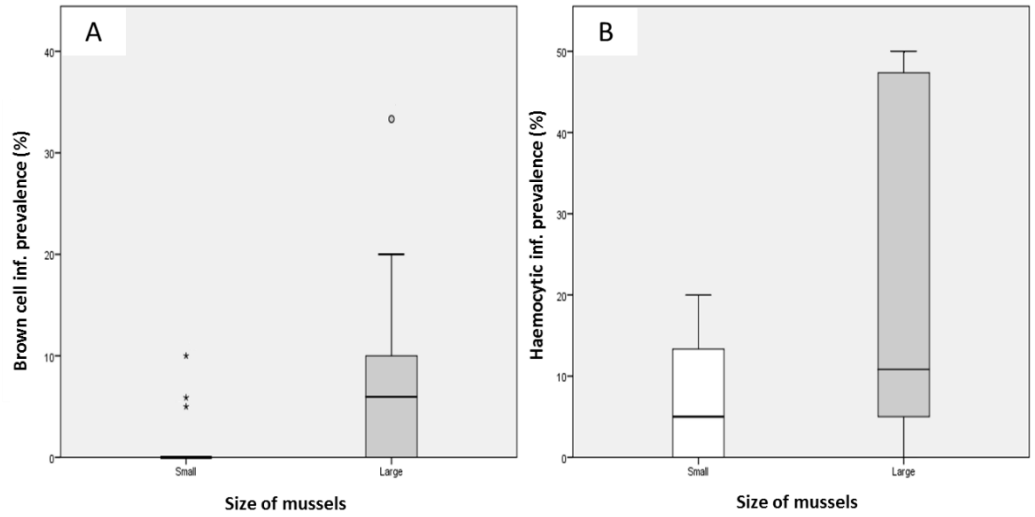

Figure 6: Changes in distribution of the prevalence (%) of brown cell infiltration (A) and haemocytic infiltration in gonad (B). Differences are statistically significant (p<0.05).

Latitudinal effects: Mussels from northernmost locations exhibited significantly higher prevalence of Gymnophallidae like trematodes (Fig. 7A) and atresia (Fig. 7C) while their counterparts from the southernmost locations exhibited significantly higher brown cell infiltrations (Fig. 7B) and granulocytoma prevalence (Fig. 7D) in the digestive gland. Mussels sampled in intermediate latitudes showed intermediate values found in-between the ones observed in north and southernmost mussels for most of the prevalences and histopathological alterations.

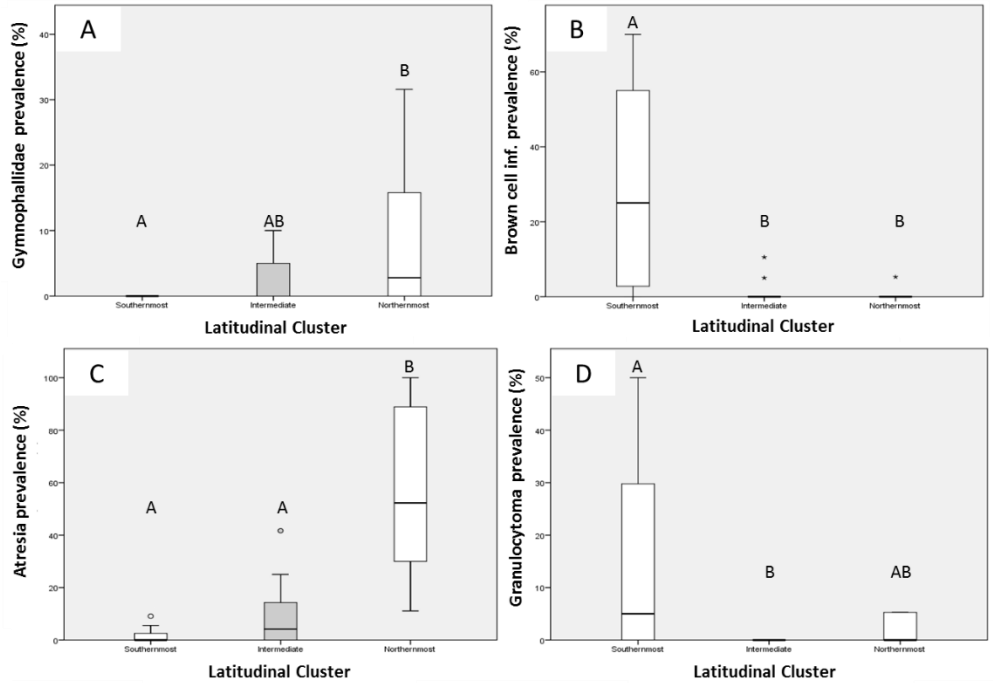

Figure 7: Changes in the prevalence (%) of Gymnophallidae like trematodes (A), brown cell infiltration in digestive gland (B), atresia of oocytes (C) and granulocytomas (D). Letters determine statistically significant differences (p<0.05).

DISCUSSION

Histopathology in mussels has been widely used as an appropriate tool for the assessment of the environmental health status because on one hand, histopathological lesions are regularly linked to exposure to pollutants and on the other hand, they can affect biomarker responsiveness (Bignell et al., 2011; Garmendia et al., 2011). In many cases, the aetiology of histopathological lesions is not related to pollutants but to other factors such as parasitism (Cuevas et al., 2015). Thus, the clarification of the causality and the potential pathogenicity are of the uttermost importance in order to interpret the aetology of the histopathological lesions correctly and avoid blaming them to chemical insult.

Reproductive status is considered one of the most important confounding factors when trying to assess the health status of a mussel population after applying biomarker approaches (Beyer et al., 2017; Blanco-Rayón et al., 2020; Cuevas et al., 2015). The spawning and post-spawning gonadal stages of mussels from the present study, in general, belong to a late summer spawning which could correspond to the second spawning process of the year, in agreement with previous findings in mussels from the Northern Atlantic and Arctic Oceans (Duinker et al., 2008; Fokina et al., 2018; Storhaug et al., 2019). The differences observed in the gamete developmental stages might be related to different growth rates along a latitudinal axis in the study area. For instance, it has been reported that southern mussels present faster growing rates (Handå et al., 2011), and therefore it is plausible that small mussels from Trondheim are younger mussels unable to perform a second spawning in the same year in an efficient way. At the same time, mussels sampled in northern latitudes seem to be old enough to perform a second spawning process (Handå et al., 2011). Moreover, part of the differences recorded in gamete developmental stages could be caused by the fact that samplings were performed sequentially within a month. Apart from size (or age), other factors such as pollution events or chronic releases of organic contaminants can trigger spawning processes due to an improved trophic condition (Dumas et al., 2020; Preisner et al., 2021), explaining the more advanced gonadal stages that were observed in mussels sampled in the vicinity of a WWTP in Trondheim. In any case, the use of mussel

shell length as a proxy of age when trying to compare mussel populations with very different ecological conditions and latitudes, could lead to wrong interpretations assuming same life stages (Izagirre et al., 2014).

Among the parasites found in the present study, trematodes are the ones that are considered to produce stronger pathogenic effect in molluscan populations (Villalba et al., 1997). Presently, the prevalence of sporocysts of trematodes (Bucephalidae) were relatively low, although the infection intensity ranged from medium to high, since the infestation reached to different organs such as digestive gland, gills and gonad. In contrast, Renicolidae-like trematodes were widespread in all latitudes along the northern Atlantic Ocean and also in Tromsø. Nevertheless, an immune reaction from the host against the parasite was appreciated in most of the cases. Moreover, significant correlations were detected between trematode presence and granulocytomas (clearly seen in mussels from Germany). The presence of trematodes might be causing important inflammatory lesions or the presence of granulocytomas could be indicative of a diminished immune system causing an increased infection rate of Renicolidae-like trematodes. However these thoughts require further research. Lastly, Gymnophallidae-like trematodes were only found in Iceland and Tromsø, which could be related to the distribution/presence of a final avian host (Galaktionov et al., 2015; Goater, 1993) that limited the presence of the parasite in other sampling sites. Interestingly, although the presence of a Gymnophallidae family trematode has previously been reported in mussels sampled in Connecticut (Galimany et al., 2008) with associated inflammatory responses and even with the presence of pearls, the description of the specific histological alteration produced by this parasite was not given. One of the main observed characteristics of the responses elicited by the parasite is the appearance of pearls. These pearls were first detected in the present study during the dissection and sectioning of histological samples from mussels infected with the trematode (data not shown). Similar host reactions against Gymnophallidae parasites were described by Kinne in 1983 in *Cerastoderma edule.* They observed that recently attached *Gymnophallus minutus* metacercariae was surrounded by columnar epithelium cells from the pallial line and once the trematode was totally enclosed by the epithelium, it started lysing the parasite. Similarly, in the present work, cellular debris is also observed

together with the presence of haemocytes in the surrounding tissues. Thus, the response elicited by Gymnophallidae-like trematode is, as far as we know, the first thorough description in *Mytilus* species. Mussels showing Gymnophallidae-like trematodes, often presented pathologies in the mantle such as atresia of the oocytes and haemocytic infiltrations in the gonad follicles or surrounding connective tissue. Pearson's test confirmed the correlation between the presence of the parasite and the haemocytic infiltration in gonad. Thus, the presence of Gymnophallidae-like trematodes as intermediate hosts in mussels is worth to take into account in health status assessment of mussels, which could have implications in marine pollution monitoring.

The second type of metazoan parasites found are the *Mytilicola sp.* copepods. The pathogenicity is considered to be low although under certain circumstances (low food availability, spawning processes…) the condition index of mussels can be affected (Pérez Camacho et al., 1997). In the present study, no important pathogenic effect was detected in relation to *Mytilicola sp.* infection. However, the presence of this parasite is conditioned by the sampling site characteristics and correlated with Renicolidae-like trematodes prevalence, as it will be discussed below.

Regarding the analysis of parasitic microorganisms, the prevalence and infection intensity of RLO/CLO was relatively low and similar to previous reports. RLO/CLO are common parasites in mussels and although they can cause hypertrophy and degeneration of the infected cells that can be fatal (Garmendia et al., 2011). It is concluded that the impact on the present mussel populations is low since no histological alterations that could indicate pathogenicity were detected.

As far as MPX parasites are concerned, the infection prevalence was in line with what has been described in the U.K. in previous studies (Fichi et al., 2018), while the infection intensity was in every case below 50 parasites per slide, which has previously been considered as a moderate infection (Gombac et al., 2008). No host response was detected in mussels infected with MPX intracellular ciliates, so the harmfulness of this parasite seems to be low or non-existent in the present work, which is concordant with previous studies (Fichi et al., 2018; Gombac et al., 2008; Villalba et al., 1997).

The histological analyses of the mussel gonads found three evident pathologies: brown cell infiltration in gonad follicles, atresia of the oocytes and haemocytic infiltration in the gonadal follicles and mantle tissue. The presence of brown cells in gonad follicles and atresia of oocytes are indicators of ongoing autolysis and resorption processes during gametogenesis, and can be induced when environmental conditions become unfavorable for spawning after gamete maturation (Katarzyna Smolarz et al., 2017; Suárez et al., 2005). Thus, these alterations were expected to occur more commonly in individuals with a more advanced gametogenic status (Cuevas et al., 2015) as in the case of mussels from Svalbard. Brown cell and haemocytic infiltration in gonad follicles were commonly found in groups where the sum of mussels in spawning and post-spawning stages was high and it was more likely to be related with the resorption of the remaining gametes and reproductive tissue. On the other hand, the histological analyses of the digestive gland found the following inflammatory responses: brown cell infiltration in digestive cells, haemocytic infiltration in digestive or connective tissue (also in gills) and the presence of granulocytomes. Brown cell infiltration in digestive tissue is probably related to the depuration process after inflammatory episodes (Bignell et al., 2011), and the presence of brown cells in male gonad follicles could also be indicative of some kind of distress causing the gametes to be reabsorbed, as discussed before. The fact that the prevalence of brown cell infiltration in digestive tissue is correlated with the prevalence of brown cell infiltration in gonad and with granulocytoma might indicate an ongoing systemic immune response. Moreover, the granulocytoma prevalence is also correlated to haemocytic infiltration in digestive gland, which could indicate a worrying pathological status in those mussel groups with the highest prevalence (large mussels from the clean sites in Germany and Scotland, and from the impacted site in Scotland). Haemocytic infiltrations are often related to the presence of pollutants, starvation processes, parasitic infection and spawning stress (Garmendia et al., 2011). Thus the high haemocytic infiltration prevalence found in mussels from different sites should be taken into account as the aetiology of the lesion can be completely different (Cuevas et al., 2015). Referring to the characteristics of the sampling sites and the presence of concurrent parasites and pathologies, in the present study the haemocytic infiltration cases might have been caused by anthropogenic impact (small and large mussels from the impacted sites in Oslo and Iceland) or by

parasitism (mussels from the WWTP in Trondheim and mussels from the clean site in Iceland), although other physiological and environmental causes cannot be discarded. The case of mussels from Scotland and Germany seems specially worrying since the high prevalence of haemocytic infiltration is accompanied (at least in large mussels) by a high presence of granulocytomas which are inflammatory responses resulting in vascular occlusions that have been linked to chronic stress (Lowe & Moore, 1979). A similar pathological status has been described in mussel beds or cultures that have suffered from high mortality episodes, like the ones described in the Netherlands in 2015/16 and 2019 (Capelle et al., 2021) where up to 70% of the mussels presented one or multiple granulocytomas. Similar episodes have been described in France since 2014 (Charles et al., 2020a). Although the cause of these episodes is unclear, the presence of a bacteria responsible for previous mortality episodes in other bivalves (*Francisella halioticida*) has been confirmed in granulocytomas found in mussel populations suffering from mortality episodes in France (Charles et al., 2020b). Although the severe histopathological condition presented by mussels from Scotland and Germany might be caused by many unknown factors, the similarities with previously described mortality episodes are to be taken into account. Thus, further research and monitoring are necessary to assess the cause, the possible geographical extension and the temporal evolution of these events. Indeed, in Scotland loss of wild mussel beds were observed in late 2017 (personal communication).

The fact that no differences in the prevalence of pathologies and parasitism were found when clustering all the groups by level of anthropogenic impact indicates that in the present study this factor does not seem to be the main one rendering differences in the histopathological profile of mussel populations. Previous studies (Cuevas et al., 2015) defined the use of a histopathological index that is responsive to the level of pollutants in the environment and although a minimum confounding effect was caused by seasonal variability and by parasitosis, the index proved to be effective. However, the use of histopathological analysis as an indicator of exposure to pollutants could cause a misinterpretation of the results given the particular structure of the present work with mussels collected from a large geographical distribution and different sizes that include mussels of very different ages. Nevertheless, the lack of statistical significance does not

mean that pollution did not have an effect in any of the pathologies, since its statistical significance could have been masked by other factors. In this sense, another known confounding factor that could interfere with the assessment of the health status of mussels is where mussels are collected from (Beyer et al., 2017). When performing studies for the biomonitoring of biological effects caused by exposure to pollutants it is hardly avoidable to sample in such different substrates. For example, the principal goal of the sampling campaign was to compare the responsiveness of biomarkers between impacted (mostly harbors) and clean sites (mostly rocky shores) along a large latitudinal span. The presence of *Mytilicola sp.* and Renicolidae-like trematodes was significantly higher in mussels sampled in horizontal substrates, which is coherent with previous studies since it has been demonstrated that mussels collected from benthic intertidal beds present higher parasitic burden than the ones collected from ropes, pylons and similar vertical structures (Buck et al., 2005). In addition, the prevalence of haemocytic infiltration was also higher in mussels sampled in horizontal substrates, probably as a consequence of a higher parasitic burden in those mussels and not by the presence of pollutants as discussed before. It might be helpful to take into account the characteristics of the substrate where mussel populations come from to avoid additional confounding effects when planning biomonitoring program. Moreover, environmental monitoring protocols might indicate that mussels of comparable shell length must be sampled (Beyer et al., 2017) as size is considered a confounding factor for the interpretation of biological responses (E. Blanco-Rayón et al., 2019b). In the present study only pathologies related to the reproductive cycle (brown cell and haemocytic infiltration in gonad) displayed statistically significant differences when comparing small to large mussels, the latter presenting higher prevalence. As discussed before, these differences are probably caused by the more advanced gametogenic stages in large mussels mostly in the southernmost and intermediate localities, and thus, it is reasonable to find differences regarding biological responses against pollution, so sampling mussels of comparable size seems to be an adequate guideline.

When comparing the prevalence of parasites and pathologies with the sampling groups clustered by latitudinal location, significant differences were found in the prevalence of Gymnophallidae like trematode, which is not surprising as it was only

found in Iceland and Tromsø and could be related to the distribution of its final host. Two pathologies that were significantly more present in the southernmost sampling points are granulocytomas and brown cell infiltration in digestive tissue. It seems that their prevalence is correlated and their significantly higher presence is caused by the exceptional histopathological status in mussels from Germany and Scotland as mentioned before. Atresia was significantly more prevalent in the northernmost locations and this significant difference was probably due to its high prevalence in mussels from Svalbard, which on the other hand is coherent with the fact that those were the groups with the biggest proportion of mature gametes in accordance with their most prevalent spawning stage.

CONCLUSIONS

The new data provided here offers a prospective view of the variability and distribution of parasites, pathologies and their effect on wild mussel populations in different environments along a wide latitudinal span along the Northern Atlantic and Arctic Oceans. A latitudinal gradient is clearly observed in gamete developmental stages as northern and southern mussels presented different conditions at the same period. Furthermore, the size/age relationship did not seem to be comparable latitudinally with evident differences in the reproductive cycle and the appearance of related pathologies. In addition, specific parasitic profiles ruled by latitudinal conditions and the settlement of mussels (horizontal/vertical) have been demonstrated to be of a great importance in the health condition of mussels. The present work also provides the first histological description of Gymnophallidae-like parasite causing an important pathogenic effect in Tromsø and Iceland plus the report of possible high mortality events related to a high prevalence of granulocytomas in Scotland and Germany. It can be concluded that it is necessary to perform a thorough histological analysis of mussels, as histopathological conditions are related to factors such as latitude, size/age, settlement and parasites that could compromise the environmental health status assessment in marine pollution monitoring.

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Chapter 4: Determination of latitudinal baseline levels and the responsiveness to pollution of a battery of cell and tissue level biomarkers in mussels (*Mytilus edulis*) from a wide geographical span in the Northern Atlantic and Arctic Oceans.

ABSTRACT

Mussels (*Mytilus sp.*) are widely used as sentinel organisms in pollution monitoring programs to determine the health status of coastal ecosystems using the biomarker approach. Although this approach has been proved to be useful, the interpretation of biomarkers related to pollution can be biased due to the interactions between chemicals and natural factors or between natural and physiological factors. Therefore, these possible confounding factors must be precisely identified. This study aims to reveal the effect of natural variability (latitude-related environmental factors and age of the collected animals) in the responsiveness of a battery of cell and tissue-level biomarkers towards pollution in mussels of two size-classes (2-3 cm and 3.5-4.5 cm) collected in relatively non-impacted sites and potentially impacted sites as harbors and a waste water treatment plant in late summer of 2017 along a wide latitudinal span in the northern Atlantic and Arctic Oceans. This work offers novel information on cell and tissue-level biomarker baseline levels and responsiveness to pollutants along a wide latitudinal span in the North Atlantic and Arctic Oceans having into account the possible confounding factors that might cause a misinterpretation of the environmental health status in the sampled areas. Despite the lack of responsiveness in certain endpoints under given environmental and physiological conditions (age and reproductive status) the battery of biomarkers of different levels of biological organization ensure the detection of differential stress signals even when important non-chemical stressors are present as in the case of the remarkable parasitism case mussels from Iceland and Tromsø suffer.

LABURPENA

Muskuiluak (*Mytilus sp*.) oso erabiliak dira kutsaduraren jarraipen programetan organismo sentinel gisa kostaldeko ekosistemen osasun egoera zehazteko biomarkatzaileen ikuspegia erabiliz. Hurbilketa hau erabilgarria dela frogatu bada ere, kutsadurarekin lotutako biomarkatzaileen interpretazioa partziala izan daiteke produktu kimikoen eta faktore naturalen edo faktore naturalen eta fisiologikoen arteko elkarreraginengatik. Beraz, nahaste faktore posible hauek zehazki identifikatu behar dira. Ikerketa honek aldakortasun naturalak (latitudearekin erlazionatutako ingurumen faktoreak eta bildutako animalien adina) zelula eta ehun mailako biomarkatzaileen bateria batek bi tamaina klasetako (2-3 cm eta 3.5-4.5 cm) muskuiluetan kutsaduraren aurrean duen erantzun gaitasunean duten eragina agerian utzi nahi du, 2017-ko uda amaieran kutsadurarik gabeko eta potentzialki kutsatutako guneetan (portuak eta hondakin uren araztegia) Ipar Ozeano Atlantiko eta Ozeano Artikoan tarte latitudinal zabal batean zehar bildutako muskuiluetan. Lan honek informazio berria eskaintzen du zelula eta ehun mailako biomarkatzaileen bateria baten oinarri balioei eta kutsatzaileen aurrean duen erantzun gatasunari buruz, lagindutako ingurumen osasun egoeraren interpretazio okerra eragin dezaketen faktore nahasgarri posibleak kontuan hartuta. Baldintza ekologiko eta fisiologiko jakin batzuetan (adina eta ugaltze egoera) biomarkatzaile batzuk kutsadurarekiko erantzunik erakutsi ez bazuten ere, antolakuntza biologiko maila ezberdinetako biomarkatzaileen bateriak estres seinale diferentzialak detektatzea bermatzen du, lan honetan bezala, estres sortzaile ez kimiko garrantzitsuak daudenean ere, Islandia-ko eta Tromsø-ko muskuiluek pairatzen duten parasitismo kasu nabarmenaren kasuan, adibidez.

INTRODUCTION

Mussels (*Mytilus sp.*) are widely used as sentinel organisms in pollution monitoring programs to determine the health status of coastal ecosystems using the biomarker approach (Brenner et al., 2014; Nasci et al., 2002). Although this approach has been proved to be useful, the interpretation of biomarkers related to pollution can be biased due to the interactions between chemicals and natural factors or between natural and physiological factors. Therefore, these possible confounding factors must be precisely identified (Benito et al., 2019; Beyer et al., 2017; Fokina et al., 2018; Leiniö and Lehtonen, 2005a; Nahrgang et al., 2013). Furthermore, the establishment of the range of natural variability and how it might influence on the correct interpretation of the biological effects caused by chemical insult is critical (Beyer et al., 2017; Izagirre et al., 2008). Although they show obvious responses to pollutants, biomarkers have displayed certain variability due to biological and environmental changes that happen naturally (Benito et al., 2019; Beyer et al., 2017; Depledge, 2009; Fernández et al., 2010; Nahrgang et al., 2013). Mussels suffer biochemical, metabolic or physiological changes which might act as confounding factors that alter organisms' capacity to respond to the presence of contaminants. Consequently, there is a need for deeper understanding of the biological cycles influencing them and the baseline levels of the biomarkers in order to correctly interpret biomarker responsiveness (Beyer et al., 2017; Nahrgang et al., 2013; Storhaug et al., 2019).

Components of marine ecosystems are susceptible of being disturbed by high concentrations of contaminants released from anthropogenic sources. The concentrations of PAHs, PCBs and metals in European coastal waters are mostly decreasing where monitored, but the amount of data is scarce in some areas, including the Norwegian Sea (EEA, 2018). North Atlantic and Arctic Oceans contain noteworthy amount of undiscovered oil and gas reserves (Gautier et al., 2009), the threat of oil spills is blooming and their hazardous ecological and socio-economic consequences are relevant to be studied. Moreover, Arctic ecosystems are highly vulnerable to oil spills due to their peculiar environmental conditions (low temperature of seawater and, more remarkably, the presence of ice-cover) and remoteness. These features could modify

the chemical composition of the spill products and intensify the toxicity to marine biota (Nordam et al., 2017; Word, 2014) and they can be major factors hampering clean-up operations after oil spills.

Mussel are present in a wide distribution in boreo-temperate region, in the North-Pacific, North- and Mid-Atlantic up to the Arctic Ocean (Kijewski et al., 2011). Their reproduction and growth has been shown to be dependent on temperature and on food availability (Berge et al., 2005; Beyer et al., 2017; Page and Hubbard, 1987; Sprung, 1983; Stirling and Okumuş, 1995; Storhaug et al., 2019; Thorarinsdóttir and Gunnarsson, 2003). Furthermore, Arctic ecosystems are characterized by drastic climatic variability In terms of light, temperature and food availability on several scales, from daily changes to seasonal and annual ones (Tran et al., 2020). Consequently, there can be a misinterpretation of biomarker responses due to the potential confounding factors related with geographic and latitudinal parameters, age and reproductive conditions, the responsiveness of biomarkers against pollution being compromised. Hence, due to the unique characteristics of the study-area the need to decipher how the natural variability of these confounding factors can affect biomarker responses it is clear (Bellas et al., 2014; Beyer et al., 2017; Bignell et al., 2008; Cuevas et al., 2015). Thus, it is of utmost importance to establish the baseline levels of biomarkers and to understand the natural variability of the biological responses to be able to properly evaluate environmental insult under a potential chemical spill.

This study aims to reveal the effect of natural variability (latitude-related environmental factors and age of the collected animals) in the responsiveness of a battery of selected cell and tissue-level biomarkers towards pollution in mussels of two size-classes collected in relatively non-impacted sites and potentially impacted sites as harbors and a waste water treatment plant in late summer of 2017 along a wide latitudinal span in the northern Atlantic and Arctic Oceans.

MATERIAL AND METHODS

Sampling, sampling processing and biomarker analysis

Sampling campaign is described in chapter 3. Samples from Germany and Scotland were not used in this chapter.

Sample processing, adipogranular cell index, cellular biomarkers and tissue-level biomarkers were performed as described in chapter 1.

Chemical Analysis of PAHs and metals

Chemical analysis of PAHs was accomplished as described in chapter 2. Chemical analisis of metals was performed as described in chapter 1.

Statistical analysis

Statistical analysis was performed as described in chapter 2.

RESULTS

Chemical burden in soft tissues

As shown in the previous case, the concentration of light and heavy PAHs and the ratio F(F+P) are shown in Figure 1. As can be seen, the highest total concentrations of PAHs are found at the polluted sites of Iceland, Tromso and Trondheim and the lowest ones at the control sites of Svalvard and Oslo. In this particular case, it is worth mentioning that the ratio HW/LW is systematically higher than 1.5 but in most of the sites even above 2.0, which would suggest a clear pyrogenic sources in all the sites. This fact is also observed in the values of the F/(F+P) rations, which are between 0.28 to 0.69 but mostly above 0.4, which would also suggest a major contribution of combustion processes. Nonetheless, the control sites of Iceland, Svalvard and Tromso show values

below 0.4 while the controls at Trondheim and Oslo show values around or above this threshold value.

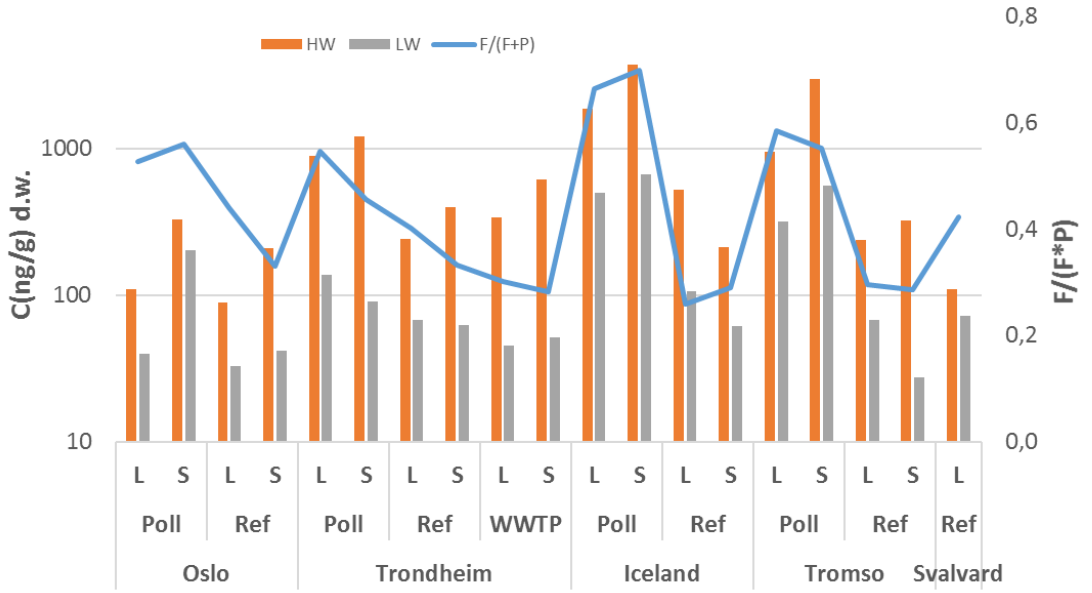

Figure 1: Plot of the light and heavy PAHs and the fluoranthene-pyrene (F/(F+P) ratios for the mussels tissues. HW: Heavy molecular weight. LW: Light molecular weight. L: Large mussels. S: Small mussels.

The PCA analysis of centered and scaled data reveals certain patterns as shown in Figure 2. On the one side, up to 53% of the total variance is explained by the first two PCs and on the other side, the samples collected ports can be broadly differentiated from those collected at reference sites except for the mussels collected in Oslo. This pattern can be interpreted in terms of the the distribution of measured PAHs and metals (supplementary materials). According to this pattern, the samples coming from the ports show an overall higher levels of PAHs, especially Phen and Ant, and Pb and Hg, among metals. On the contrary, the samples collected at the reference sites would show higher levels of Ni, V or As.

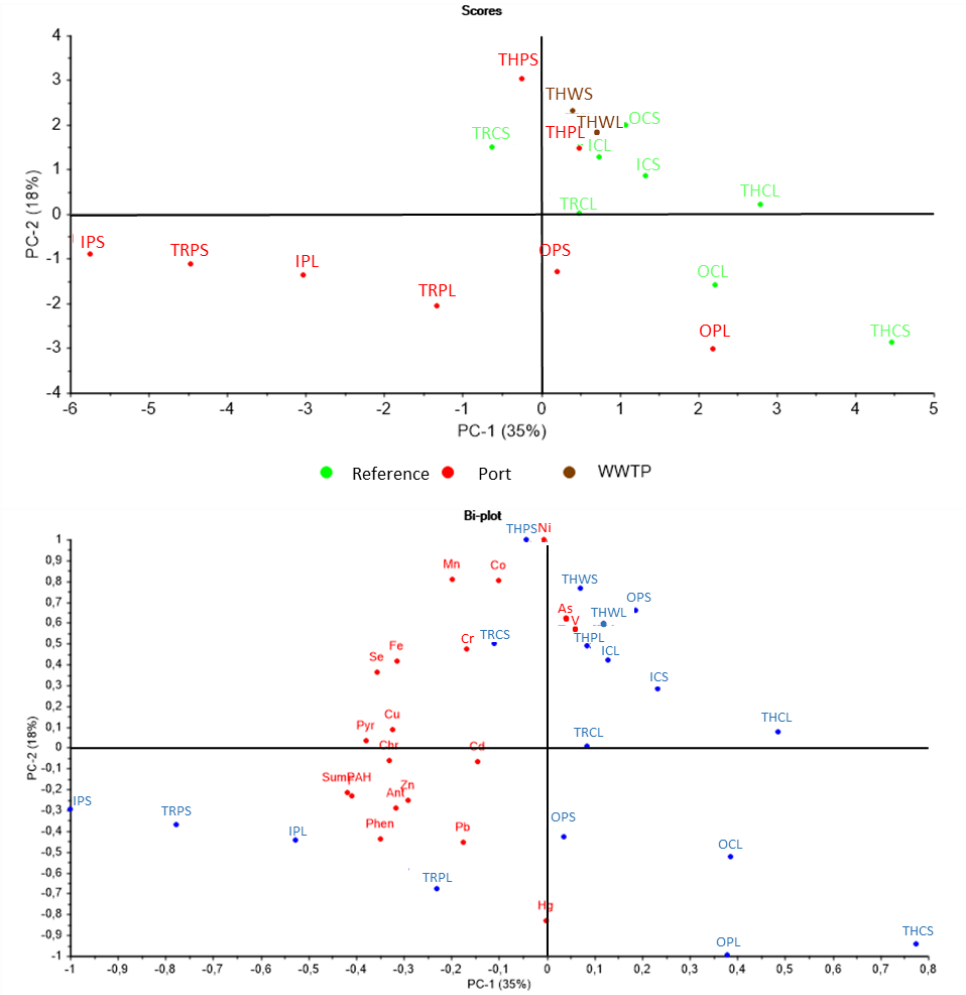

Figure 2: Plot of the 2 Principal Component Analysis measurements explaining the 53% of the total variation of the sampled groups caused by the concentration of pollutants in soft tissues of mussels. O: Oslo, TH: Trondheim, I: Iceland, TR: Tromsø, S: Svalbard. C: Reference site, P: Port, W: WWTP.

Adipogranular cell index

ADG index values (Figure 3) were lower in small mussels from the reference site in Iceland when compared to the rest of same sized mussels from the reference sites except mussels from Svalbard. Large mussels from the reference site in Trondheim presented significantly higher ADG values when compared to large mussels from the reference sites in Iceland and Svalbard. No significant differences were found marked by the presence of pollutants. Small mussels from the reference site in Oslo, small mussels from the three sites in Trondheim and small mussels from both sites in Iceland displayed lower ADG values when compared to the large mussels from the same sites.

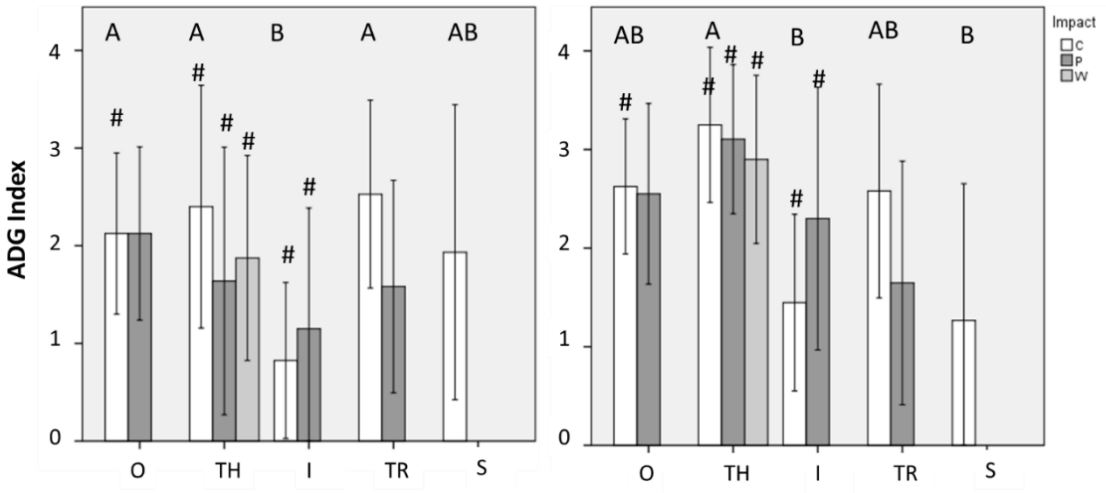

Figure 3: Adipogranular cell (ADG) index in mantle tissue. Left: small mussels, right: large mussels O: Oslo, TH: Trondheim, I: Iceland, TR: Tromsø, S: Svalbard. C: Reference site, P: Port, W: WWTP. Capital letters mean statistical differences among reference sites. Hash mean statistical differences among mussel sizes in the same site.

Cellular biomarkers

Vv_{LPF} (Figure 4) showed no significant differences among small mussels. Large mussels only showed significant differences among the reference site from Tromsø and Svalbard, the latter presenting higher values. In addition, no significant differences were found between reference and polluted sites and sizes.

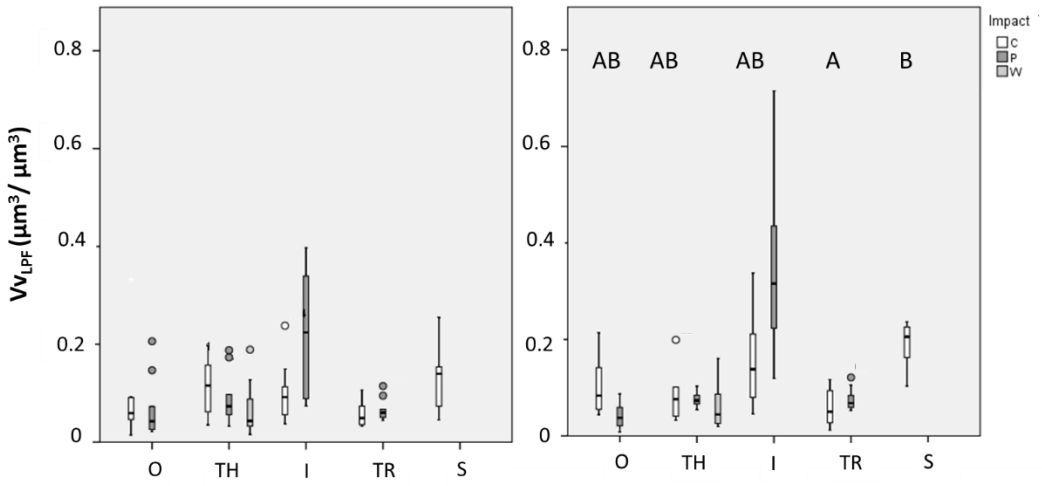

Figure 4: Lipofuscin volume density (Vv_{LPF}). Left: small mussels, right: large mussels O: Oslo, TH: Trondheim, I: Iceland, TR: Tromsø, S: Svalbard. C: Reference site, P: Port, W: WWTP. Capital letters mean statistical differences among reference sites.

Vv_{NL} values (Figure 5) in small mussels only varied significantly among reference sites. Icelandic mussels presented highest values, while mussels from Trondheim and Tromsø presented intermediate values. Lowest values were detected in mussels from Oslo and mussels from Svalbard. Among large mussels, reference sites in Trondheim, Iceland and Tromsø presented significantly higher values when compared to reference sites in Oslo and Svalbard. No significant differences were found regarding effect of size and pollution.

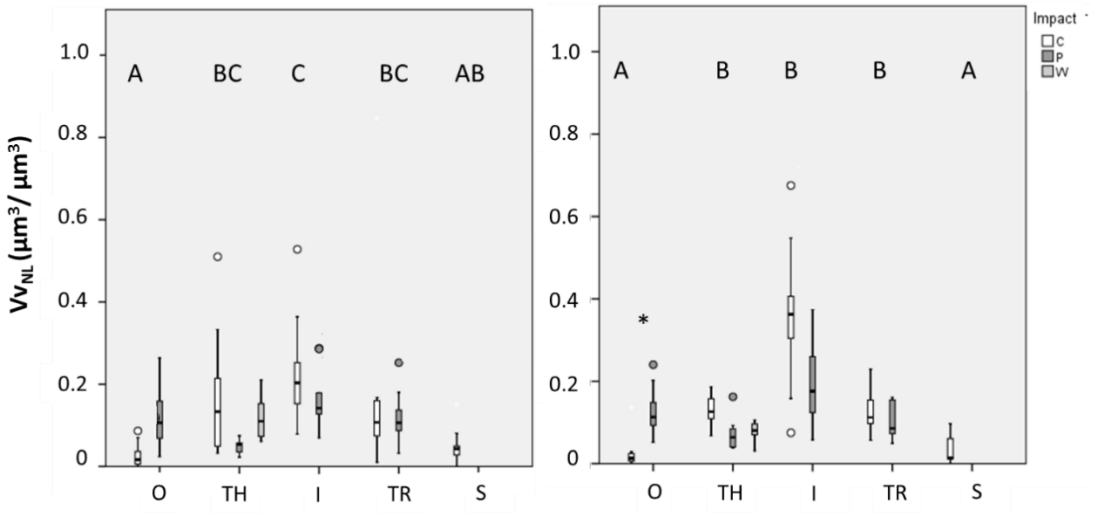

Figure 5: Neutral lipid volume density (Vv_{NL}). Left: small mussels, right: large mussels O: Oslo, TH: Trondheim, I: Iceland, TR: Tromsø, S: Svalbard. C: Reference site, P: Port, W: WWTP. Capital letters mean statistical differences among reference sites. Asterisks indicate statistically significant differences between reference and impacted sites in the same locality.

Regarding LMS (Figure 6), small mussels from the ports in Trondheim, Iceland and Tromsø presented significantly lower lysosomal labilisation periods when compared to the small mussels from the reference site in the same locality. Large mussels from the ports in Iceland and Tromsø displayed significantly lower labilisation periods when compared to large mussels from the reference sites in the same localities. Small mussels from the reference site in Oslo showed significantly higher labilisation periods when compared to large mussels in the same sampling sites. No significant differences were found when comparing reference sites.

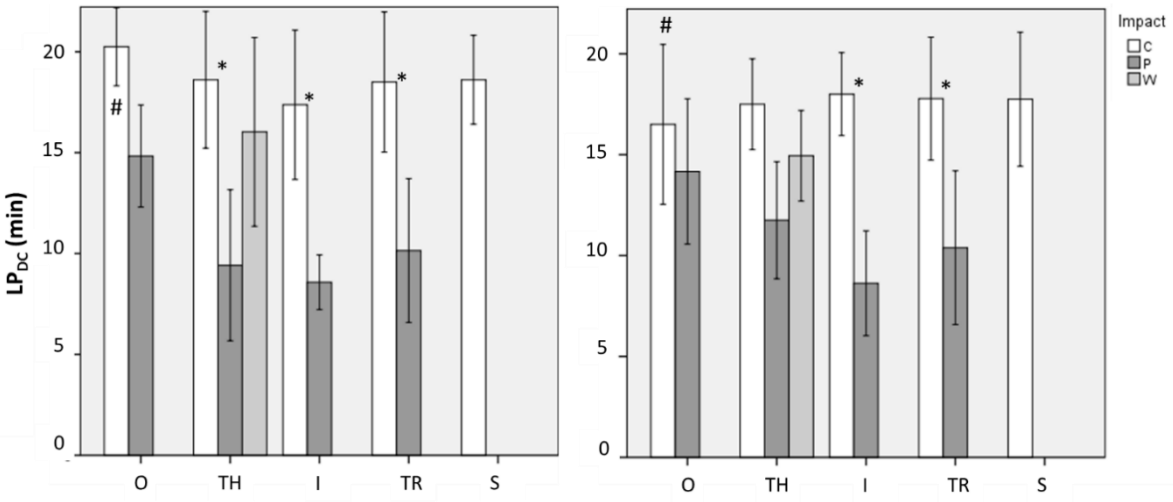

Figure 6: Labilisation period (LP, min) measured in lysosomal membrane stability test. Left: small mussels, right: large mussels O: Oslo, TH: Trondheim, I: Iceland, TR: Tromsø, S: Svalbard. C: Reference site, P: Port, W: WWTP. Hash mean statistical differences among mussel sizes in the same site. Asterisks indicate statistically significant differences between reference and impacted sites in the same locality.

LSC could not be reliably measured in Iceland and Svalbard. Vv_{LYS} (Figure 7) in small mussels was significantly lower in mussels from the reference site in Oslo when compared to the mussels from the reference sites in Trondheim and Tromsø. Small mussels from the reference site in Trondheim showed significantly higher values than mussels from the port in the same locality, while mussels from the WWTP presented intermediate values. S/V_{LYS} in small mussels presented differences among the reference sites, being the mussels from Oslo the ones presenting the highest values. Nv_{LYS} in small mussels was significantly higher in mussels from the reference site in Oslo when compared to the ones from the reference sites in Trondheim and Tromsø, furthermore, mussels from the reference site in Oslo presented significantly higher Nv_{LYS} values than mussels from the port in Oslo. Vv_{LYS} values in large mussels presented significant differences when comparing reference sites, as mussels from the reference site in Tromsø showed significantly higher values. Mussels from the port in Oslo presented significantly higher Vv_{LYS} values when compared to the mussels from the reference site in the same locality. S/V_{LYS} and Nv_{LYS} values were significantly higher in large mussels from the reference site in Oslo when compared to the other reference sites and when compared to the large mussels from the port in Oslo. The only differences between mussel size-classes in the same sampling site were detected in the reference site in Trondheim were small mussels presented significantly higher Vv_{LYS} and in mussels from the reference site in Oslo where small mussels displayed significantly higher Nv_{LYS} values.

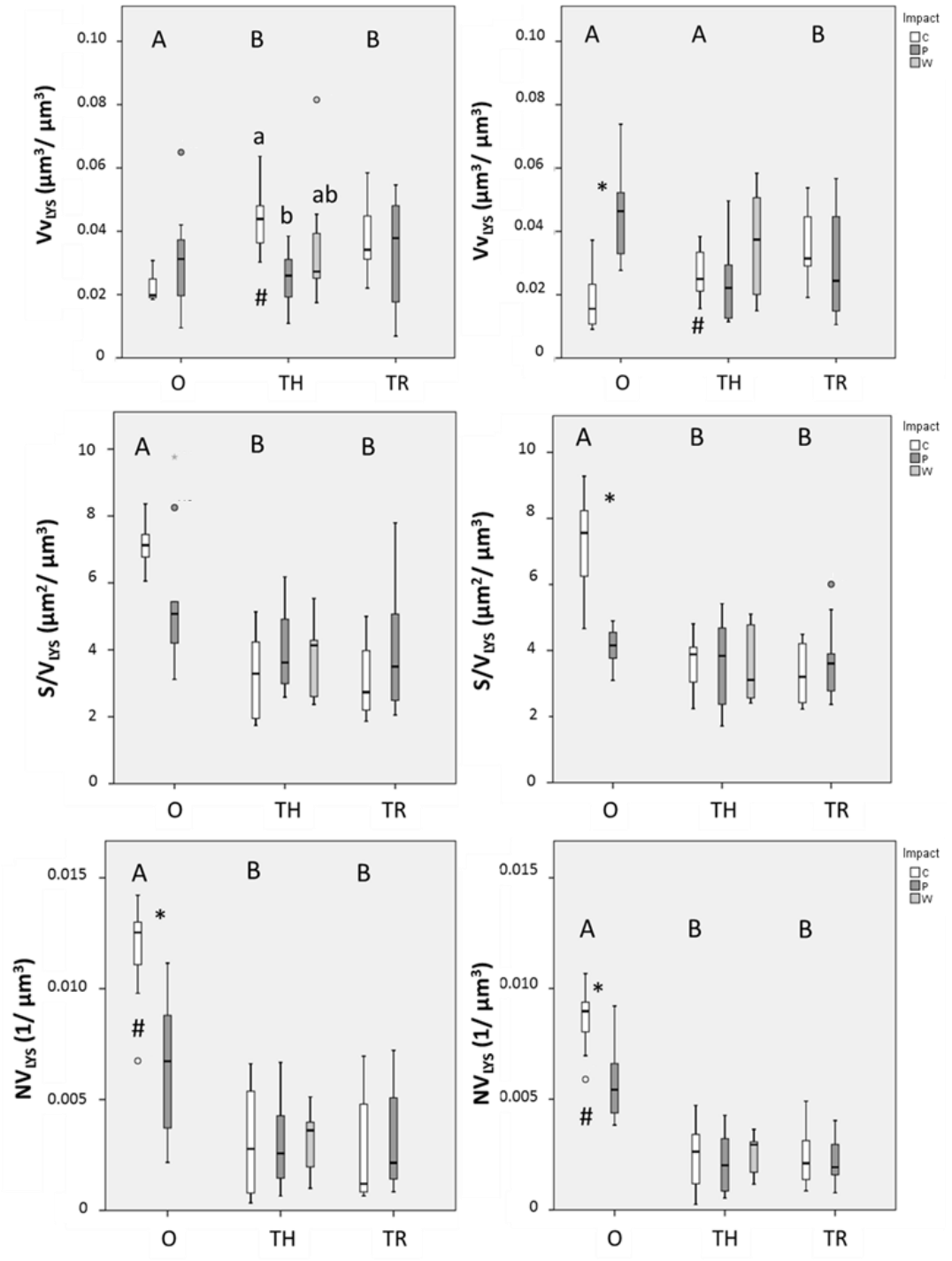

Figure 7: Lysosomal volume density (Vv_{LYS}) (top), surface to volume ratio (S/V_{LYS}) (middle) and numeric density (Nv_{LYS}) (bottom). Left: small mussels, right: large mussels O: Oslo, TH: Trondheim, TR: Tromsø. C: Reference site, P: Port, W: WWTP. Capital letters mean statistical differences among reference sites. Small letters indicate statistical differences between sampling sites in Trondheim. Asterisks indicate statistically significant differences between reference and impacted sites in the same locality. Hash mean statistical differences among mussel sizes in the same site.

Small mussels from the reference site in Trondheim presented significantly lower Vv_{BAS} values when compared to small mussels from the WWTP, while mussels from the port presented intermediate values. Large mussels from the reference sites in Oslo and

Tissue level biomarkers

Trondheim displayed significantly lower Vv_{BAS} values (Figure 8) when compared to mussels from the reference sites in Iceland, Tromsø and Svalbard. Large mussels from the reference site in Trondheim presented significantly lower Vv_{BAS} values than same sized mussels from the harbor and WWTP in Trondheim. Large mussels from the reference site in Iceland also presented significantly lower Vv_{BAS} values than the same sized mussels from the port in the same locality. In addition, small mussels from the reference sites in Oslo and Trondheim and small mussels from port in Trondheim presented significantly higher Vv_{BAS} values than the large mussels from the same sites.

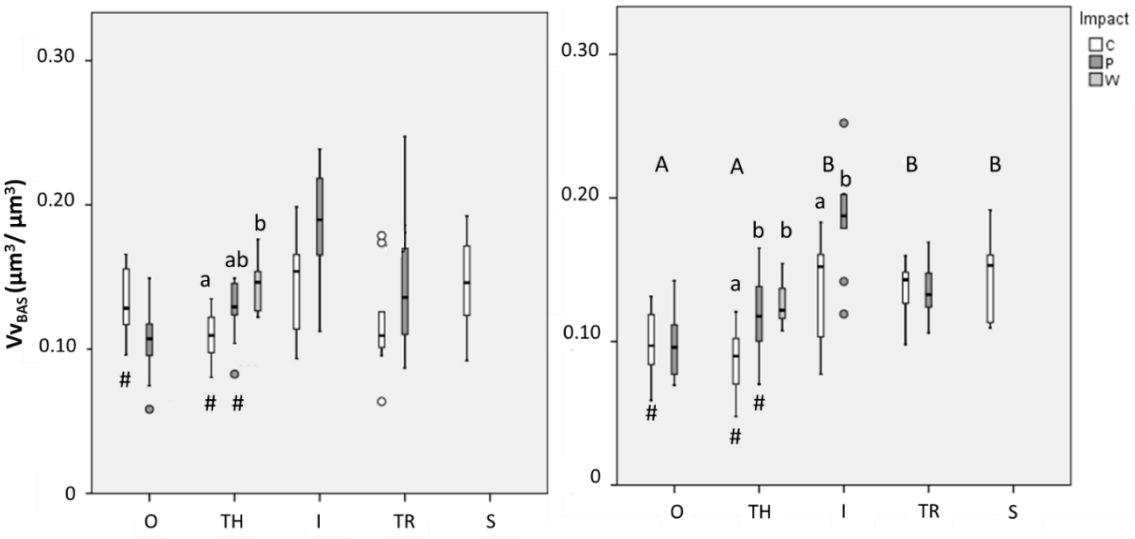

Figure 8: Basophilic cell volume density (Vv_{BAS}). Left: small mussels, right: large mussels O: Oslo, TH: Trondheim, I: Iceland, TR: Tromsø, S: Svalbard. C: Reference site, P: Port, W: WWTP. Capital letters mean statistical differences among reference sites. Small letters indicate statistical differences between sampling sites in Trondheim. Hash mean statistical differences among mussel sizes in the same site.

Small mussels from the reference site in Oslo displayed significantly higher CTD values (Figure 9) when compared to the rest of small mussels from the other reference sites. Large mussels from the reference site in Oslo presented significantly higher CTD values when compared to large mussels from the reference site in Iceland, while the rest of large mussels from the reference sites displayed intermediate values. Small mussels

from the harbor in Iceland displayed significantly lower CTD values compared to large mussels in the same sampling site.

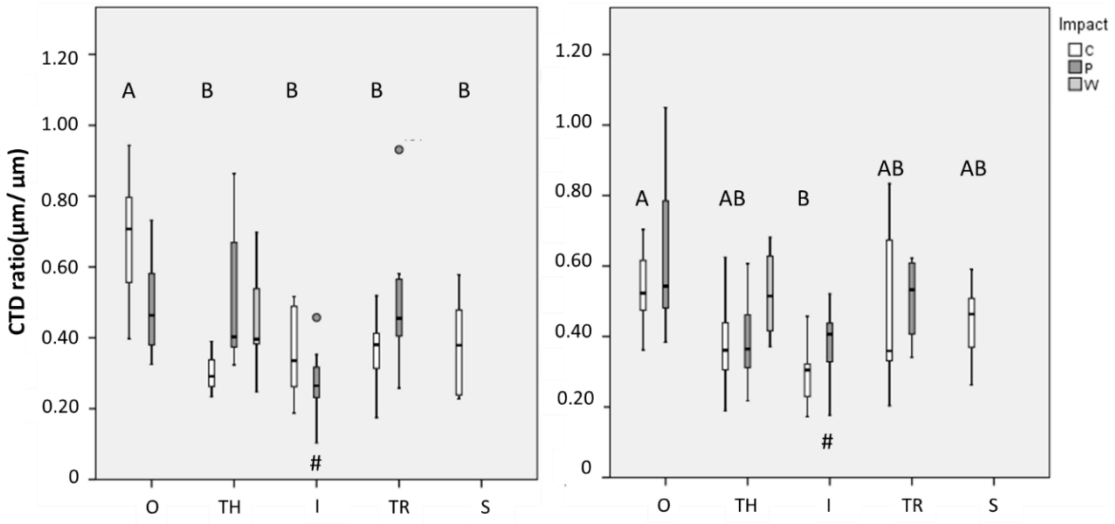

Figure 9: Connective to digestive tissue (CTD) ratio. Left: small mussels, right: large mussels O: Oslo, TH: Trondheim, I: Iceland, TR: Tromsø, S: Svalbard. C: Reference site, P: Port, W: WWTP. Capital letters mean statistical differences among reference sites. Hash mean statistical differences among mussel sizes in the same site.

Atrophy index values (Figure 10) were significantly higher in small mussels from the reference site in Oslo when compared to same sized mussels from the reference sites in Trondheim, Iceland and Svalbard. Small mussels from the reference site in Tromsø presented intermediate values. Small mussels from the reference site in Trondheim displayed lower atrophy index values when compared to small mussels from the port in Trondheim, while small mussels from the WWTP showed intermediate values. Atrophy index values in large mussels from the reference sites in Oslo and Tromsø were significantly higher than the ones presented by mussels from the reference sites in Trondheim and Iceland. Atrophy index values in mussels from the reference site in Trondheim were significantly lower when compared to mussels from the port and the WWTP in the same locality. Small mussels from the port in Iceland and Tromsø and small mussels from Svalbard presented lower atrophy index values when compared to large mussels from the same sites.

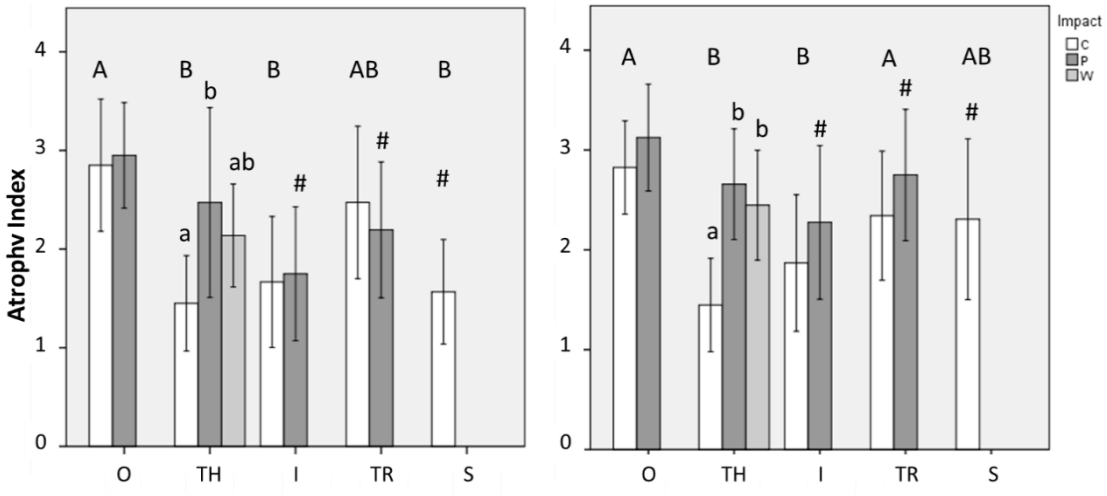

Figure 10: Atrophy index. Left: small mussels, right: large mussels O: Oslo, TH: Trondheim, I: Iceland, TR: Tromsø, S: Svalbard. C: Reference site, P: Port, W: WWTP. Capital letters mean statistical differences among reference sites. Small letters indicate statistical differences between sampling sites in Trondheim. Hash mean statistical differences among mussel sizes in the same site.

DISCUSSION

The present study provides an approach to revealing the responsiveness to pollution of a battery of selected biomarkers in mussels sampled along a wide latitudinal span in the Northern Atlantic and Arctic Oceans to assess environmental health taking into account biomarker variability caused by latitude-related environmental factors and physiological (age and reproductive stage) status. The knowledge of the influence of these confounding factors is crucial to correctly assess the impact caused by a potential environmental disaster (e.g. oil spill) in cold waters and to design efficient biomonitoring programs along a latitudinal gradient with marked environmental differences.

The average total concentration of the 16 PAHs is around 1000 ng/g (dry weight) and ranges from 120 to 4400 ng/g with a remarkable contribution of heavy PAHs, especially Fluoranthene (F) and Pyrene (Pyr). Since most of the reference EQS values are given in wet weight basis (e.g. for F and P, the reported values are 30 ng/g, which would mean 5 times higher in dry weight). Nonetheless, we can compare to the results reported by Poulsen et al. (2021) from West Greenland but from less impacted sites and with those reported by Elskus et al. (2020) from the Gulf of Maine (USA). Though the

sum of the PAH16 reported in the former work ranges from 25 to 286 ng/g (dw), the pollution pattern also suggests combined petrogenic and pyrolytic sources. On the contrary, the values reported in the second work show a much wider variability with mean values ranging from < 20 ng/g (dw) to 1600 ng/g (dw). In addition, PCA analysis of chemical burden in soft tissues of mussels showed that the sites defined as reference sites in the present work are different to the ports defined as chemically impacted sites. In addition, the fact that mussels from the ports were affected by different mixtures of metals and PAHs make it expectable to find different kind of responses in certain biomarkers as it will be discussed below.

The primary energy reserves used for gametogenesis are usually accumulated in ADG cells, and thus the variation in their abundance typically follows a seasonal cycle related to gametogenesis. Deviations from this seasonal variation in the ADG cell density are normally considered as indicators of contaminant exposure and physiological stress (Bignell et al., 2011). ADG cell density was affected by the size of mussels being lower in the reference ones from Oslo and in all mussels collected in Trondheim and Iceland. This differences could be related with different gonadal stages described in chapter 3, as large mussels in southern regions presented more advanced gametogenic stages than small mussels. Other factor that can affect this dissimilar values is the age of mussels, the fact that small mussels in the southern localities could be younger than the ones in the northern locations which could imply different physiological status (Duinker et al., 2008), being this caused by different growth rates at different latitudes (Handå et al., 2011). Coherently, mussels from the less impacted sites in Tromsø and Svalbard did not show such differences among sizes. The only significant ADG index difference between reference sites among small mussels was found in Iceland, where the lowest values found in this work could be related to sampling site specific trophic and/or environmental condition as described in mussels from the Baltic Sea (Benito et al., 2019). On the contrary the significantly higher ADG index values found in large mussels from the reference site in Trondheim when compared to mussels form Iceland and Svalbard, could be explained by a better trophic condition in mussels from Trondheim taking into account that they present similar gametogenic stages, which is coherent with what has been previously discussed for small mussels. Unlike, large mussels from

Svalbard, which were in the an ongoing spawning phase, exhibited low ADG values (Moukrim et al., 2008). These results are to be taken into account as they could be an accurate indicative of the physiological differences among mussels of different sizes and/or sites that can condition biomarker responsiveness, as it will be discussed later on.

Lipofuscin content presented high inter-individual variability as it could be expected when comparing mussels subdued to different trophic regimes, tides and chemical insult among other environmental variables. It has been reported that Vv_{LPF} is susceptible of changing depending on oxidative stress (Viarengo et al., 1991), but also when certain environmental conditions like long-term food deprivation in winter in the Baltic Sea followed by a subsequent spring bloom (Benito et al., 2019) affect mussels. Dietary variations have also caused differences in laboratory conditions in previous studies (E. Blanco-Rayón et al., 2019a). Thus, low Vv_{LPF} in mussels from the reference site in Tromsø cannot be directly linked to a better environmental status, although it seems to be consistent in mussels from that sampling site, as it was described also in the 2nd chapter.

The lowest neutral lipid contents in the digestive cells of mussels were found in the reference sites in Oslo and Svalbard. In mussels from Svalbard the generalized ongoing spawning process could explain the low Vv_{NL} levels, suggesting a mobilization of lipid reserves for reproduction (Aarab et al., 2011; Guerlet et al., 2007). In mussels from Oslo the same explanation could be valid for large mussels, but not in small ones, as the percentage of post-spawning mussels is below 30% (chapter 3), so probably the low Vv_{NL} values are caused by environmental factors such as bad dietary condition, as discussed below. Vv_{NL} content in large mussels from the reference site in Oslo were significantly lower than in the harbor, which could be caused by the fact that mussels from the harbor were subtidal with continuous food availability and were not subjected to the stress caused by the low tide period (Sprung, 1983; Storhaug et al., 2019). The results of the present study indicate that there were no significant differences between small and large mussels in terms of neutral lipids accumulation in the digestive gland. It can be concluded that the effect of size/age can be neglected and probably environmental factors are the ones governing this endpoint as suggested by the

differences among reference sites in the present work. Previous studies in the Bay of Biscay (Garmendia et al., 2010) reported reference Vv_{NL} values of 0.05-0.1 µm3/ µm3 in October which is only comparable with the results obtained in the reference site in Oslo, the harbor in Trondheim and mussels from Svalbard in the present work. Mussels from the rest of the sampling sites displayed Vv_{NL} values that were higher or similar to the higher part of the Bay of Biscay range while mussels from the reference site in Iceland were way above the described range. These results are concordant with previous works (Brooks et al., 2015) and they confirm that northern *M. edulis* naturally display higher Vv_{NL} values than the mussels in the Bay of Biscay.

Previous studies have reported the responsiveness of LMS to chemical insult (Izagirre and Marigómez, 2009). In the present study small mussels from the ports in Trondheim, Iceland and Tromsø presented significantly lower labilisation periods when compared to the reference sites in the same locality. Similarly, large mussels from the ports in Iceland and Tromsø presented significantly lower labilisation periods than the reference sites in the same locality. Although it has been reported that labilisation periods in small mussels tend to be lower due to enhanced digestive activity related to higher metabolic rate and growth (Izagirre et al., 2014). In the present study no differences were found between sizes except in mussels from the reference site in Oslo, where small mussels presented higher labilisation periods. This difference could be related to the high percentage of large mussels that were in post-spawning and spawning (almost 90%), while small mussels from the same site only presented 20-30% of individuals in post-spawning. It is known that changes in gametogenic stages can alter the lysosomal responses as part of the seasonal variability described in mussels (Izagirre et al., 2008). In addition, as discussed before, mussels in the port of Oslo were subtidal, so the lack of tidal stress and continuous feeding probably caused the mussels to be able to cope with pollution more efficiently. Threshold levels in mussels from the bay of Biscay are stablished in >20 minutes for pristine environmental conditions (Marigómez et al., 2013), but in the present study the reference sites and the WWTP present values that are between 10-20 minutes which correspond to tolerable environmental condition. Mussels sampled in the harbors (except mussels from the harbor in Oslo) are, on the other hand, in the range of delicate environmental condition (5-10 min). These

results are in concordance with previous studies that compared the response of certain biomarkers in Norwegian *M. edulis* with Basque *M. galloprovincialis* (Brooks et al., 2015), where slightly lower LP were measured in Norwegian mussels compared to Basque mussels. Coherently with the 2nd chapter, it seems that the LMS thresholds stablished in *M. galloprovincialis* from the Bay of Biscay should be used as reference with caution when working with *M. edulis* in northern latitudes.

Small mussels from the reference site in Oslo presented lower Vv_{LYS} values with smaller lysosomes in higher numbers when compared to small mussels from the reference sites in Trondheim and Tromsø, which could be concordant with Vv_{NL} differences described before. Both biomarkers could be altered by geographical and/or physiological variables which could cause a certain natural variability in this endpoints and a potential confounding effect (Izagirre et al., 2008). Small mussels from the port in Trondheim presented lower Vv_{LYS} when compared to small mussels from the reference site, which could be concordant with the responses to metal pollution in Norwegian *M. edulis* as reported by Brooks et al. (2015) or to the low concentration of organic contaminants (Etxeberria et al., 1994). Large mussels from the reference sites in Oslo and Trondheim presented lower Vv_{LYS} when compared to large mussels from the reference site in Tromsø, which differs from the results described in small mussels from the same sites. The reason could be the different gametogenic stages in small and large mussels from the reference site in Trondheim as the latter presents a bigger portion of spawning individuals which might cause a physiological stress that could alter the lysosomal structure (Benito et al., 2019; Izagirre et al., 2008). The same factors might be influencing the significantly larger lysosomes in large mussels from the port in Oslo compared to large mussels from the reference site in the same locality, as the more advanced gametogenic stage in mussels could cause an additional stress that summed to the mild pollution could exert some stress responses at low levels of biological organization. The differences found between the two sizes of mussels from the reference site in Trondheim, where small mussels presented higher Vv_{LYS} could be related to higher metabolic and feeding rates in younger mussels, as discussed before. The lack of differences between both sites in mussels from Tromsø could be a mixture of mild pollutant levels in the harbor and the confounding effect of the Gymnophallidae

like trematodes found in this locality as discussed in chapter 2 and 3. Maximum values registered in the current study regarding Vv_{LYS} are above the threshold values described in the Bay of Biscay for bad environmental condition (Marigómez et al., 2013), furthermore, the highest values are displayed by mussels from the reference sites in Trondheim and Tromsø, while mussels from the reference site in Oslo presented comparable results. The implications of these results may be important and we are not able to explain the reason behind with the information of the present research work. In any case, it cannot be discarded the seasonal or geographical effects (Benito et al., 2019). Interestingly the present data shows slightly lower Vv_{LYS} in Trondheim and Tromsø than what it is reported in chapter 2, which could indicate certain seasonal effect (Izagirre et al., 2008). Nevertheless it is remarkable that previous studies (Brooks et al., 2015) reported higher Vv_{LYS} values in *M. edulis* than in *M. galloprovincialis*, even though the values reported in the present work are considerably lower than the ones referenced.

In general terms cell level biomarkers showed partial responsiveness to pollution in the sampled sites. Lipofuscin content in the digestive cells of mussels did not present identifiable trends, probably derived from the high susceptibility to be altered by confounding factors this endpoint displays. Neutral lipid accumulation on the other hand presented predominant geographical and physiological differences that overcame the effect of the pollutants present in the impacted sampling sites. On the contrary, LMS seemed to be a clear indicator of chemical insult, except for mussels in Oslo where the physiological differences between sizes (age) and the fact that mussels from the impacted site were subtidal caused altered responses. LSC showed responsiveness to pollution but it was affected by size (age) of mussels in Oslo and Trondheim and by parasitism in Tromsø, in addition, the reasons causing the unreliable measurements in mussels from Iceland and Svalbard require further research.

The only differences found regarding cell type composition in digestive alveoli in small mussels were found in Trondheim, where mussels from the WWTP presented higher. This is probably more related to the stress generated by the presence of digenean parasites and reproductive effort (chapter 3) summed to the stress generated by the light chemical burden found in soft tissues of mussels from that site. Regarding

large mussels, the ones from the reference sites in Oslo and Trondheim displayed significantly lower Vv_{BAS} values when compared to mussels from the reference sites in Iceland, Tromsø and Svalbard, which could be caused by parasitism (chapter 3) in the first two localities (Cuevas et al., 2015; Garmendia et al., 2011) and by the predominant spawning process in the latter (Benito et al., 2019). As discussed in the 2nd chapter, in previous studies mussels from Trondheim (Brooks et al., 2011) presented higher values than the mussels from the Bay of Biscay (Marigómez et al., 2013). Although in the 2nd chapter no significant differences were found between mussels from the reference sites in Tondheim and Tromsø, the differences found in the present dataset could be related with the fact that the sampling was performed earlier in the year when mussels of different sites/sizes presented different gametogenic status, which could heavily affect biomarker responsiveness (Benito et al., 2019). Large mussels from the harbor and WWTP in Trondheim and from the harbor in Iceland presented higher Vv_{BAS} values than large mussels from the reference sites in the same localities, which is probably caused by the presence of pollutants in the harbors (E. Blanco-Rayón et al., 2019c). The stress response measured in large mussels from the WWTP might be caused by the combined impact of mild concentration of pollutants and the parasitic burden (Chapter 3) found in that site. In the present study Vv_{BAS} seemed to be one of the endpoints showing more variability between size classes in southernmost locations, which is in concordance with the different gametogenic stages described in chapter 3, and with the fact that in southernmost locations small mussels are younger mussels with different physiological and digestive activities, which is coherent with ADG index results. Thus, the possibility of slight differences appearing when sampling different sized mussels must be taken into account. The lowest values in the present study were similar or above 0.1 $\mu m^3/ \mu m^3$ which is the good environmental condition threshold in the Biscay Bay (Marigómez et al., 2013). Thus it could be concluded that the Vv_{BAS} baseline values and its responsiveness towards pollution for northern *M. edulis* mussels are higher than the ones stablished for mussels from the Bay of Biscay, being this concordant with the 2nd chapter and the data reported in previous studies (Brooks et al., 2011).

CTD ratio presented high interindividual variability and the only significant differences were found when comparing mussels from different reference sites. Small

mussels from Oslo presented higher CTD ratio values than the rest of reference sites of the same size. In the case of large mussels the only significant differences were the higher CTD ratio values found in mussels from Oslo when compared to mussels from Iceland. The high CTD ratio in small mussels from Oslo is concordant with the high atrophy index and together with the low Vv_{NL} values could be indicative of poor dietary condition (Benito et al., 2019; Esther Blanco-Rayón et al., 2019). Furthermore, the slightly higher stress signals present in small mussels from the reference site in Oslo compared to large mussels from the same site could be linked to the significantly higher ADG index values found in the latter. As discussed before these signs might be indicative of a more demanding physiological status in small mussels despite of the fact that a larger fraction of the large mussels were in a post-spawning phase. The significantly higher CTD values found in large mussels from the port in Iceland when compared to small mussels from the same site could be related to the higher number of large mussels presenting an ongoing spawning process, as it has been described that under certain conditions reproductive effort can affect CTD ratio heavily (Benito et al., 2019).

As discussed before mussels from the reference site in Oslo and Tromsø (and large mussels in Svalbard) presented high atrophy levels. In the case of mussels from Oslo it could be caused by unknown environmental factors, although as it has been discussed earlier, bad dietary condition is concordant with the relatively high atrophy levels and other stress responses. The high atrophy index values in mussels from Tromsø could be caused by the presence of the Gymnophallidae like trematodes found in the reference site (chapter 3) and as described in the 2nd chapter these parasites elicited stress responses in several biomarkers. Regarding the responsiveness of the atrophy index to pollution, significant changes were only found in Trondheim in mussels of both sizes as mussels from the port presented higher atrophy index values when compared to mussels from the reference site. Large mussels from the WWTP presented significantly higher atrophy index values when compared to large mussels from the reference site in Trondheim, which could be caused by a combination of mild pollution and the presence of Renicolidae like trematodes (chapter 3) that could act as background stressors (Stier et al., 2015; Thieltges, 2006). Opposed to the results obtained from the majority of the biomarkers, large mussels from the ports in Iceland

and Tromsø and large mussels from Svalbard presented higher atrophy index values than their small counterparts. As discussed earlier, small mussels in the northernmost locations are supposed to be older and thus they should have less physiological and reproductive differences when compared to large mussels from the same sites. Although age might not be the factor influencing the differences found in the epithelial thickness which has been described to be affected by dietary condition (Esther Blanco-Rayón et al., 2019), body size could be the factor governing these differences as it has been reported that small mussels present relatively higher feeding and ingestion rates, which would cause lower assimilation efficiency under high food concentrations (Widdows, 1978).

Summarizing, tissue level biomarkers seemed to present less geographical variability and their responsiveness to pollution was mostly affected by parasitism and physiological factors, which enforces the need of applying a battery of biomarkers of different biological complexity.

CONCLUSIONS

The present study gives novel information on cell and tissue-level biomarker baseline levels and responsiveness to pollutants along a wide latitudinal span in the North Atlantic and Arctic Oceans having into account the possible confounding factors that might cause a misinterpretation of the environmental health status in the sampled areas. Despite the lack of responsiveness in certain endpoints under given environmental and physiological conditions (age and reproductive status) the battery of biomarkers of different levels of biological organization ensure the detection of differential stress signals even when important non-chemical stressors are present as in the case of the remarkable parasitism case mussels from Iceland and Tromsø suffer. Further research will include the increase of the number of biomarkers of lower levels of biological complexity and data integration methods in order to cover a wider array of responses and to give a holistic point of view of the complex responses obtained.

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4. EZTABAIDA OROKORRA, ONDORIOAK, TESIA.

EZTABAIDA OROKORRA

Ikerlan honen ekarpen nagusia zelula eta ehun mailako biomarkatzaile bateria baten lehenbiziko aplikazioaren latitude Artiko eta Subartikoetan muturreko ingurumen baldintzak jasaten dituzten muskuiluetan (*Mytilus sp.*) azterketa da. Honek, lagindutako muskuiluei eragiten dieten nahaste faktore histopatologiko, fisiologiko eta ekologikoen detekzioa eta biomarkatzaileen oinarri balioen eta erantzun gaitasunaren identifikazioa ahalbidetu du, ingurumen osasun egoeraren ebaluazio integratua burutu ahal izateko balio dezakenak.

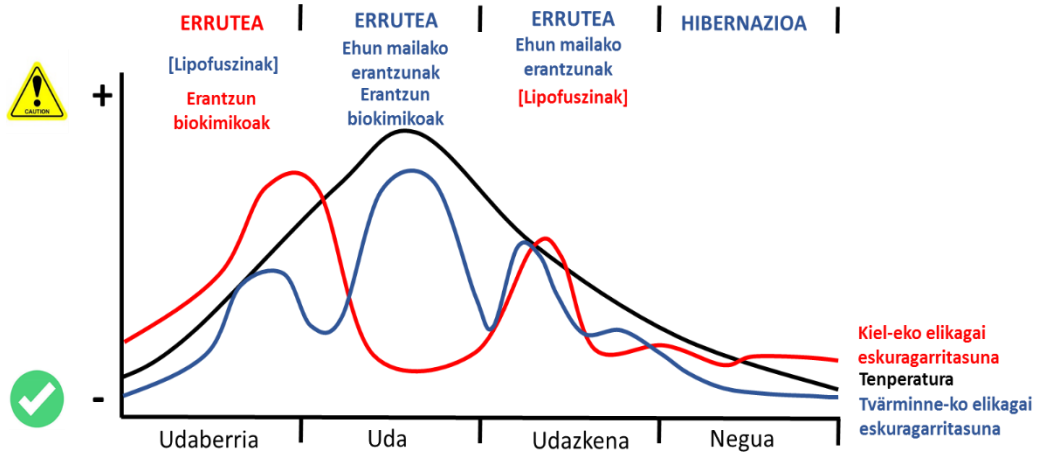
Bizkaiko Golkoaren osasun egoera adierazteko hainbat biomarkatzaile eta horien oinarri balioak erreferentzia moduan erabili dira (Garmendia et al., 2010; Marigómez et al., 2013). Eredu hau jarraiturik muturreko baldintzak (latitude artiko eta subartikoak) dituzten ekosistemen osasun egoera aztertzeko biomarkatzaile bateria berdinaren oinarri balio eta erantzun gaitasun identifikatzeko ahaleginak egin dira ikerlan honetan.

Dena dela, helburu hau era egokian betetzeko ikertutako eremu bakoitzean laginketa gehigarriak nahitaezkoak suertatzen dira. Adibidez, Itsaso Baltikoaren kasuan (1. atalean) nahiz eta aukeratutako bi eremu geografikoetan eta urtaroetan zehar eman ziren ingurugiro baldintzen aldaketak nahiko ondo zehaztea lortu zen, aldagarritasun handiko esparru geografiko horren baldintzak direla eta, laginketa puntu gehiagoetan hurbilketa berdina errepikatzeak, modu sistematikoan, ekosistemaren osasun egoerari buruzko informazio kritikoa eta zehatzagoa emango luke. Horretaz gain, urtarokotasuna hobeto ulertzeko laginketaren maiztasun handiagoa (adb. hilabetero) onuragarria izango litzateke, Leiniö eta Lehtonen (2005)-ek bertan egindako ikerketa batean proposatu zuten antzera. Ipar ozeano Atlantikoan egindako ikerketetan (2. eta 4. atalak) berriz, aldakortasun geografikoa era estentsiboan landu zen arren, kokapen geografiko bakoitzean urtaroekin aldatu ziren ingurugiro baldintzek biomarkatzaileen erantzun gaitasunean zuten ondorioa ezezaguna da, 2. eta 4. Ataletan Norvegiako Trondheim eta Tromsøn lagindutako muskuiluetan aurkitu ziren gonadaren eta hainbat biomarkatzaileen desberdintasun nabariak argi uzten zuten moduan. Gainera, kontuan eduki behar da biomarkatzaileen bitartez egindako ikerketa batean lortutako emaitzak

eskualde mailan adierazgarriak izateko egokia izango litzatekela kilometro gutxiagoren baitan laginketa puntu gehiago aztertzea, Storhaug et al. (2019)-ek proposatu zuten laginketa-estrategia jarraituz. Dena den, gure ikerlan honetan ikertutako eremu geografikoetan orain arte neurtu ez ziren biomarkatzaileak erreferentzia ahalmenik gabe geratu ez zitezen, Itsaso Baltikoan oso erabiliak izan ziren biomarkatzaile biokimikoak (Lehtonen et al., 2016; Leiniö and Lehtonen, 2005b; Schiedek et al., 2006; Turja et al., 2014) ere ikertu ziren (1. atala), eta Ipar Ozeano Atlantikoan lagindutako muskuiluetan (2. eta 4. atalak) etorkizunean ikertuak izango dira.

Kutsadurak aukeratutako biomarkatzaileetan sortutako aldaketak orokorrean ondo identifikatu dira ikerketa honetako atal desberdinetan. Hala nola, Itsaso Baltikoari dagokiola, nahiz eta zelai ikerketan (1. atala) eremu kutsatuetatik muskuilurik ezin izan zirenez bildu, ez dago aukeratutako zelula eta ehun mailako biomarkatzaileen (LMS, LSC, LPF, NL, Vv_{BAS}, atrofia indizea, CTD) portaeraren zelai eredurik.

Bestalde, Ipar Ozeano Atlantikoan egindako ikerketetan (2. eta 4. atalak) leku garbiez gain leku kutsatuetako muskuiluak ere lagindu eta aztertu ziren. Eremu kutsatuetako muskuiluen ehun bigunetan metatutako kutsatzaileen kontzentrazioak ez ziren izugarri altuak bibliografian aurkitutako balio orokorrekin konparatuta (Beyer et al., 2017; Robinson et al., 2017), baina biomarkatzaileetan estres erantzunak sortzeko nahikoak izan ziren. Adibidez, deskribatutako LMS balio orokorrean baxuagoak portuetan eta Vv_{BAS} balio altuagoak kutsadura maila altuenak erakusten zituzten portuetan. Dena den, esan beharra dago deskribatutako faktore nahasgarri anitzen eragina zela eta biomarkatzaileen erantzun guztiz homogeneoa ez zela behatu.



12. Irudia: Itsaso Baltikoan egindako zelai ikerketaren (1. atala) laburpen grafikoa.

Aldaketa histopatologikoek Ipar Ozeano Atlantikoan lagindutako muskuiluetako erantzun biologikoetan (antolakuntz maila desberdinetako biomarkatzaileen bidez lortuak) eragin oso garrantzitsua zeukatela ere ikusi da 2., 3., eta 4. ataletan. Esate baterako, 3. atalean ondorioztatu zen bezala, muskuiluetan oinarritutako biojarraipen programa baten laginketa puntuak aukeratzerakoan garrantzitsua izan daiteke hauen ezaugarri fisikoak eta muskuiluen kokapena kontuan edukitzea lesio histopatologikoak konparagarriak izan daitezen eta faktore nahasgarri bezala jokatu ez dezaten. Lesio histopatologikoen artean kasu deigarrienak Eskozia eta Alemaniako muskuiluetan aurkitu ziren granulozitoma prebalentzia altuak eta Islandia eta Tromsø-n aurkitu diren Gymnophallidae antzeko trematodoak dira.

3. atalean eztabaidatu zen moduan, granulozitomen prebalentzia altuak Europako leku askotan muskuiluen hilkortasun altuko gertakariekin erlazionatuta egon daitezke, analisi histopatologikoak duen garrantzia azpimarratzen duenak. Interesgarriki, eta erlazio hori (muskuiluen hilkortasuna eta granulozitomen prebalentzia altua) konfirmatuko balitz, Euskal kostaldean azkeneko urteotan antzeman den bertoko muskuiluen populazioaren gainbeherakada (behaketa pertsonala) azaldu zezakeen, atariko analisi histopatologikoek granulozitomen presentzia altua konfirmatu baitute Euskal kostaldeko kokapen jakin batean. Erantzun gabe dago zer nolako faktore probokatu zuen efektu hori. Kutsadura kronikoa? Berotze globalaren ondorioz parasitoen larbak neguan zeharreko biziraupen handiagoa? Beraz, granulozitoma eta hilkortasun gertakari hauek sortzen dituzten patogeno edo ingurugiro baldintzak definitzea ikerketa bide berria izan daiteke epe motzera.

Aipatutako Gymnophallidae antzeko trematodoaren inguruan ere prozesuan dauden aurretiazko froga molekularrak egin dira parasitoa molekularki identifikatzeko eta muskuiluetan “perlak” sortarazten dituztela molekularki konfirmatzeko. Dena hau, 3. atalean deskribatutako hedapen geografiko zabaleko parasitoen ikerketa filogenetikoak, laser mikrodisektore bitartez egiteko asmoa dugu hurrengo hilabeteotan.

Ikerlan honetan zehar bete diren helburuak eta antzeman diren ahultasunak kontuan edukita, Itsaso Baltiko eta Ipar Ozeano Atlantikoko ekosistemen osasun egoeraren ebaluazio sakonagoa eta zehatzagoa egin ahal izateko biomarkatzileen

gaineko faktore nahasgarriak identifikatu dira azpimarratu daiteke. Etorkizuneko zelai ikerketetan eta laborategiko esperimentuetan landutako ikerketa eremuetako muskuiluen erantzun biologikoak ulertzeko tesi honek erreferentzia material erabilgarri moduan balioko du, ekosistema artiko eta subartikoen osasun egoeraren ezagutza areagotzen jarraitzeko eta munduan barreneko beste esparru geografikoekin konparaketak egin ahal izateko.

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ONDORIOAK

1- Itsaso Baltikoan egindako zelai azterketak *Mytilus trossulus* muskuiluen bertako populazioak ingurumen baldintza ezberdinen eraginpean egotea posible dela (tenperatura, gazitasuna, elikagaien erabilgarritasuna) leku desberdinetako muskuiluetan biomarkatzaileen erantzun desberdinak erregistratzean argitzen du. Beraz, funtsezkoa da urtaroen efektuak eta itsaso Baltikoko leku desberdinetako biomarkatzaileen oinarri balio naturalei eragiten dieten ingurumen faktore desberdinak eta gorabeheratsuak ezagutzea. Leku bakoitzean aldagai ekologikoen eta baldintza fisiko kimikoen karakterizazioa funtsezkoa da Itsaso Baltikoan kutsaduraren ondorioen ebaluazio fidagarria egiteko.

2- 2016-an Ipar Ozeano Atlantikoan egindako kanpaina pilotua zelula eta ehun mailako biomarkatzaileen oinarri balioak eta kutsatzaileen aurrean erreakzionatzeko duten gaitasuna ezartzeko lehen urratsa da Norvegiako itsasoko latitude artiko eta subartikoetan dauden bi tokietan (Trondheim eta Tromso). Nahiz eta lan honetan erabilitako biomarkatzaileen bateria baliagarria izan den inpaktatu eta inpaktu gabeko muskuilu populazioak bereizteko, erantzun biologikoak aldatzen dituzten nahaste faktore batzuk identifikatu ziren (alterazio histopatologikoak, parasitoen prebalentzia, garapen gametogenikoa). Oro har, lortutako emaitzek aingura puntu gisa balio dute, bai aztertutako eremu geografikoan eta urteko garaian aztertutako biomarkatzaileen oinarri balioen erreferentzia gisa, bai estresa eragiten duten ingurumen faktore nahasgarrien hedadura potentzialaren adierazgarri gisa.

3- Analisi histopatologikoek parasitoen, patologien eta haien efektuen aldakortasunaren eta banaketaren ikuspegi prospektiboa eskaintzen dute ingurune ezberdinetako muskuilu populazioetan Ipar Ozeano Atlantiko eta Ozeano Artikoan tarte latitudinal zabal batean zehar. Hala ere, kontuan hartu beharreko muga batzuk daude, beraz, muskuiluen analisi histologiko sakon bat egitea beharrezkoa da baldintza histopatologikoak latitudeak, tamainak/adinak, muskuiluak finkatuta dauden substrato mota eta parasitoak baldintzatzen baidituzte, kutsatzaileen jarraipen programetan ingurumenaren osasunaren ebaluazioa arriskuan jar dezaketen faktoreak baitira.

4- Lan honek muskuiluetan (lehen berberetxoetan deskribatuak izan ziren) Gymnophallidae-itxurako parasitoen lehen deskribapen histologikoaren berri ematen du, Tromsø-n eta Islandian efektu patogeno garrantzitsua eragiten dutenak. Gainera, Eskozian eta Alemanian granulozitomen prebalentzia altuarekin lotutako hilkortasun altuko gertakari posibleen froga ere ematen da.

5- 2017-an Ipar Ozeano Atlantikoan eta Ozeano Artikoan egindako kanpainak zelula eta ehun mailako biomarkatzaileen oinarri balioei eta kutsatzaileekiko erantzun gaitasunari buruzko informazio berria ematen du tarte latitudinal zabalean zehar. Testuinguru honetan, lagindutako eremuetan ingurumen osasun egoeraren interpretazio okerra eragin dezaketen nahaste faktoreak aztertzen dira. Nahiz eta biomarkatzaile batzuek erantzun eza aurkeztu zuten baldintza ekologiko eta fisiologiko jakin batzuetan (adina eta ugalketa egoera), erabilitako konplexutasun biologiko maila ezberdinetan dauden biomarkatzaileen baterian estres seinale diferentzialak detektatzea bermatzen du, nahiz eta estres kimikoa ez den beste faktore garrantzitsuak eragin, Islandia-ko eta Tromsø-ko muskuiluetan parasitismo kasu nabarmenaren kasuan, adibidez.

TESIA

Zelula eta ehun mailako biomarkatzaile baterien oinarri balioak eta aldakortasun naturala ezartzea funtsezkoa dela frogatu da osasun egoeraren ebaluazio holistikoa eta muskuilu populazio ezberdinen artean konparazio zehatzak eta integratzaileak egiteko, *Mytilus sp.* muskuiluak espezie sentinel gisa erabiliz kutsaduraren jarraipen programa eraginkorrak diseinatu ahal izateko beharrezkoa dena.