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Challenges for shellfish aquaculture in Mediterranean coastal areas

Article based thesis

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Errata page

Abstract

The increase in the production of marine bivalves targets the sustainable development of aquaculture to improve global food and nutrition security. Bivalve aquaculture is currently facing challenges and threats that are limiting its growth and even its maintenance. Summer marine heat waves produce important mortalities endangering the harvest of the year and the seed of the following harvest. There is a need for expansion to deeper areas less affected by these events. We have developed a methodology to select these areas based on a combination of in situ and remote sensing data to build carrying capacity model for a coastal area in the Western Mediterranean. Harmful algal blooms (HABs) have an impact on bivalve aquaculture through the production of toxins that can accumulate in the bivalves affecting human health, to avoid that, shellfish closures are enforced in areas where toxins are detected over regulatory levels. Direct effects of HABs also threaten bivalve aquaculture producing mortality of farmed bivalves. We have evaluated the risk and trends of HABs in the Mediterranean Sea using bibliographic references and records of HABs events. The results show a low risk of toxic blooms but a higher risk of high biomass blooms without clear trends. Unexplained mortalities of farmed bivalves occur in the Mediterranean Sea, the presence of pathogens alone does not always explain these events. We have detected the presence of *Perkinsus olseni* for the first time in *Mytilus galloprovincialis* from the Mediterranean Sea. Warming may stimulate its proliferation, but we cannot conclude that its presence alone was the trigger of the mortality event. Translocation of bivalves from other geographical areas may be a risk for the introduction of pathogens and invasive species. In addition, the production of local seed may have other benefits due to bivalve adaptation to local environmental conditions. The replacement of live microalgae diets with artificial diets to simplify hatchery-nursery procedures has been attempted by different teams during the last decades. Artificial diets have been successful for fish and crustaceans but not yet for bivalves. We have tested the efficacy of an artificial diet to feed larvae of *Ostrea stentina*. Our results show significant differences in the growth and survival of the same larvae feed with live microalgae ponting out that it is not adequate for the hatchery and nursery production of *Ostrea stentina* larvae. We have addressed various challenges of the bivalve aquaculture in the Mediterranean Sea and have provided tools and evaluations to overcome the imitations and facilitate its development.

Key words: Bivalve aquaculture, Mediterranean Sea, Ebro delta, Carrying capacity, Remote sensing, Harmful Algal Blooms, Shellfish pathogens, *Perkinsus*, *Mytilus galloprovincialis*, Chlorophyll,

Preface

Exploring and observing the nature, we can find new ways of progress improving our lives and minimising our negative impacts.

I am grateful to all of those who shared their knowledge with me.

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List of abbreviations and symbols

C_R: Clearance rates of mussels.

$\dot{P}x$: is the ingestion rate of mussels.

AFLP: Amplified Fragment Length Polymorphism.

APD: average percentage difference

apig: pigment absorption product.

AS: Adriatic Sea.

ASC. Aquaculture Stewardship Council.

ASP: Amnesic shellfish poisoning.

Aura OMI NASA: The Ozone Monitoring Instrument (OMI) aboard NASA's Aura satellite measures ozone from Earth's surface to top-of-atmosphere (https://www.earthdata.nasa.gov/learn/find-data/near-real-time/omi).

AZP: Azaspiracid poisoning.

B01: Coastal aerosol, 442.7 nm (S2A), 442.3 nm (S2B), 60m (https://docs.sentinel-hub.com/api/latest/data/sentinel-2-l1c/).

B02: Blue band, 492.4 nm (S2A), 492.1 nm (S2B), 10m resolution.

B03: Green band, 559.8 nm (S2A), 559.0 nm (S2B), 10m resolution.

B04: Red band, 664.6 nm (S2A), 665.0 nm (S2B),10m resolution.

B05: Vegetation red edge band, 704.1 nm (S2A), 703.8 nm (S2B), 20m resolution.

B06: Vegetation red edge band, 740.5 nm (S2A), 739.1 nm (S2B), 20m resolution.

B07: Vegetation red edge band, 782.8 nm (S2A), 779.7 nm (S2B), 20m resolution.

B08: NIR band, 832.8 nm (S2A), 833.0 nm (S2B), 10m resolution.

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B11: SWIR band, 1613.7 nm (S2A), 1610.4 nm (S2B), 20m resolution.

B12: SWIR band, 2202.4 nm (S2A), 2185.7 nm (S2B), 20m resolution.

B8A: Narrow NIR band, 864.7 nm (S2A), 864.0 nm (S2B), 20m resolution.

BG: Blue to Green ratio.

BG2: Blue to Green ratio 2.

BHR: Broad host range.

BIAS. is the tendency of a statistic to overestimate or underestimate the parameter or measurement.

BLAST: Basic Local Alignment Search Tool.

BLAST: Basic Local Alignment Search Tool.

C2-Nets: Atmospheric correction processors derived from the original Case 2 Regional Coast Colour.

C2RCC: Case 2 Regional Coast Colour.

C2XC: C2X-COMPLEX processors

cal: calibration.

CC: carrying capacity.

CDOM: Colored dissolved organic matter.

CFP: Ciguatera.

CLM: Cloud masks (more) data, 160m resolution.

CLP: Cloud probability data, based on s2cloudless (more), 160m resolution.

CLUSTAL: software for multiple sequence alignments.

COI sequence: Mitochondrial cytochrome c oxidase subunit I.

CT: clearance time.

DA: Domoic acid.

DEB: Dynamic Energy Budget.

DNA: Deoxyribonucleic acid.

dNTP: Deoxynucleotide triphosphate.

DSP: Diarrhetic Shellfish Poisoning.

DTX: dinophysistoxin.

DU: Dobson units

eDNA: Environmental DNA.

EOVs: Essential Ocean Variables.

ESA: European Space Agency.

EU: European Union.

f/2 medium: this microalgae culture medium contains the concentration of the original formulation, termed "f Medium" (Guillard and Ryther 1962), reduced by half.

FSW: Filtered seawater.

GenBank: an annotated collection of all publicly available DNA sequences (https://www.ncbi.nlm.nih.gov/genbank/).

GIT: 3 band model from Gitelson et al 2011.

GTP: Graphic Processing Tool.

HAB: harmful algal bloom.

HABMAP: Harmful Algal Bloom Map.

HABs: harmful algal blooms.

HAEDAT: Harmful Algal Event Database.

HB-HABs: High biomass harmful algal blooms.

iCOR: is a software to atmospherically correct Earth observation data.

IOC: Intergovernmental Oceanographic Commission.

ITS: Internal Transcriber Spacer.

L1C: Level 1C products are a compilation of elementary granules of fixed size, within a single orbit. A granule is the minimum indivisible partition of a product (containing all possible spectral bands). The granules, also called tiles, are 100x100km^2 ortho-images in UTM/WGS84 projection. The UTM (Universal Transverse Mercator) system divides the Earth’s surface into 60 zones. Each UTM zone has a vertical width of 6° of longitude and horizontal width of 8° of latitude. Tiles are approximately 700 MB in size. Tiles can be fully or partially covered by image data. Partially covered tiles correspond to those at the edge or top and bottom of the Datastrip (Copernicus Sentinel-2 Collection 1 MSI Level-1C (L1C) , https://sentinels.copernicus.eu/web/sentinel/sentinel-data-access/sentinel-products/sentinel-2-data-products/collection-1-level-1c) Copernicus Sentinel-2 (processed by ESA), 2021, MSI Level-1C TOA Reflectance Product. Collection 1. European Space Agency, https://doi.org/10.5270/S2_-742ikth.

MAE: mean average error.

MEGA: Molecular Evolutionary Genetics Analysis.

MHW: Marine heat wave.

ML: Maximum likelihood analysis.

MLC: Maximum likelihood composite distances

MS: Mediterranean Sea.

MSI: Multispectral instrument.

MUSCLE: MUltiple Sequence Comparison by Log- Expectation. It is a software for creating multiple alignments of protein sequences.

MW: Meat weight.

NCEP/DOE Reanalysis II: The NCEP-DOE Reanalysis 2 project is using a state-of-the-art analysis/forecast system to perform data assimilation using past data from 1979 through near present. A large subset of this data is available from PSL in its original 4 times daily format and as daily and monthly averages (https://psl.noaa.gov/data/gridded/data.ncep.reanalysis2.html).

NDCI: Normalized Difference Chlorophyll Index.

NIS: Non-indigenous species.

NJ: Neighbour-joining.

NOAA: National Oceanic and Atmospheric Administration

NPZD: biogeochemical nutrient-phytoplankton-zooplankton-detritus model.

OA: okadaic acid.

OBIS: Ocean Biogeographic Information System.

OIE: Founded in 1924 as the Office International des Epizooties (OIE), in May 2003 adopted the common name World Organisation for Animal Health (WOAH).

P: Phosphorous.

PB: phytoplankton biomass

PCR: Polimerase Chain Reaction.

Pearson’s r: Pearson’s correlation coefficient, it measures the statistical relationship, or association, between two continuous variables. It is based on the method of covariance.

PopART: Population Analysis with Reticulate Trees, it is free population genetics software.

PPP: phytoplankton primary production.

PPT: number of days required for the replacement of phytoplankton biomass in a water body considering phytoplankton growth.

PSP: Paralytic Shellfish Poison

PT: time required to renew the phytoplankton stock in ana area.

PVC: Polyvinyl Chloride.

QGIS: is a professional GIS application that is built on top of and proud to be itself Free and Open Source Software (FOSS). https://qgis.org/en/site/about/index.html

qPCR: real-time PCR or quantitative real-time PCR.

rDNA: ribosomal DNA.

REB1: Red Edge 1 to Blue.

REG1: Red Edge 1 to Green.

REG2: Red Edge 2 to Green.

RER: Red Edge 1 to Red.

RFTM: Ray's Fluid Thioglycollate Medium.

RG: Red to Green ratio.

RGB: red, green and blue, it refers to a system representing the colors used on a digital display screen, these three colors may be combined in various proportions to obtain any color in the visible spectrum.

RMSE: root mean squared error.

ROMS: Regional Ocean Model System (https://www.myroms.org/).

Rrs: Remote sensing reflectances.

RT: renewal time of a water body.

S2: Sentinel 2.

SFG: Scope for growth.

SNAP: ESA's Sentinel Applications Platform (https://step.esa.int/main/download/snap-download/)

SPM: suspended particulate matter.

SST: Sea surface temperature

SYBR green: N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine, used as a nucleic acid stain.

TAADs: Transboundary aquatic animal diseases.

TCS network: is a Java computer program to estimate gene genealogies including multifurcations and/or reticulations (i.e. networks). The network estimation implemented in TCS is also known as Statistical Parsimony.

TD: transboundary diseases.

TSM: total suspended matter.

UNESCO: United Nations Educational, Scientific and Cultural Organization.

val: validation.

YTX: Yessotoxins.

ZSD: Secchi disk depth.

Tr: is the renewal time of the water in an area.

Glossary

Apoptosis: a form of programmed cell death that occurs in multicellular organisms and in some eukaryotic, single-celled microorganisms.

Arrhenius temperature function: It calculates the dependence of the rate constant of a chemical reaction on the absolute temperature.

Atmospheric correction: it is the process of removing the scattering and absorption effects from the atmosphere to obtain the surface reflectance characterizing (surface properties).

Carrying capacity: of a biological species or individuals from several species in a particular habitat refers to the maximum number of individuals (of that species or several species) that the environment can carry and sustain, considering its geography or physical features.

Case-2 waters: case 1 is that of a concentration of phytoplankton high compared to other particles. In contrast, the inorganic particles are dominant in case 2. In both cases dissolved yellow substance is present in variable amounts. An ideal case 1 would be a pure culture of phytoplankton and an ideal case 2 a suspension of non-living material with a zero concentration of pigments.

Clearance rate: volume of water cleared of suspended particles per unit of time.

Davidson solution: is a rapid fixative that contains deionised water, acetic acid, ethanol, phosphate buffered formaldehyde.

Fed aquaculture: it includes aquaculture of salmon, shrimps, trout, seabass and other species, feed often—though not always—contains wild-captured fish.

Food safety: it refers to the conditions and practices for the production, manufacturing, processing, packing, or holding that preserve the quality of food to prevent contamination and food-borne illnesses.

Food security: it exists when all people, at all times, have physical and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life". (World Food Summit, 1996).

Granulocytes: a type of white blood cell that has small granules inside them. These granules contain proteins. The specific types of granulocytes are neutrophils, eosinophils, and basophils.

Haemocytes: circulating cells with a very important role in the immune system of invertebrates, which can be found within the hemolymph, analogous to the blood in vertebrates.

Haplotype: a group of genes within an organism that was inherited together from a single parent.

Hypnospores: a very thick-walled asexual resting spore.

Low trophic aquaculture: can include unfed shellfish, seaweed and some species of finfish, and can also include fed species that primarily depend on plant products in their feeds.

Marine heat wave: a period of abnormally high ocean temperatures relative to the average seasonal temperature in a particular marine region. Marine heat waves are usually defined as any time the ocean temperature is above the 90th percentile for a specific length of time. This means that the temperatures are warmer than 90 % of the previous observations for a given time of year obtained using a climatological period (usually spanning 30 years).

Non fed aquaculture: In non-fed culture systems, fish or shellfish is completely relying on natural food present in the captive conditions to fulfil their nutritional requirement and there will not be any supplementation of pellet feed. It includes seaweed and bivalves as examples.

Perkinsiosis: commonly known as dermo disease, is caused by protozoan parasites of the genus Perkinsus.

Protandric hermaphrodite: Sequential hermaphroditism.

Restorative Shellfish Mariculture: Restorative aquaculture occurs when commercial or subsistence aquaculture provides direct ecological benefits to the environment, with the potential to generate net-positive environmental outcomes. Principles of Restorative Aquaculture: 1) Farms are sited where environmental outcomes are needed. 2) Species are cultured that can provide the environmental outcomes intended. 3) Farming equipment that enhances the delivery of environmental benefits is prioritized. 4) Management practices that align with or enhance local ecological processes are adopted. 5) The intensity and scale of culture works to enhance ecosystem. 6) The socio-economic value of the environmental benefits provided are recognized.

Sea surface temperature anomaly definition: The sea surface temperature anomaly is the difference between the analyzed surface temperature at a point on the model and the average value at that same point over a long period of time, for the same month of the year, the average value being taken from the reanalysis associated to the model.

Schnute-Richards growth model: an extended form of von Bertalanffy's growth function, it relates to growth acceleration.

Secchi disk: it is a plain white, circular disk 30 cm in diameter used to measure water transparency or turbidity in bodies of water. The depth at which the disk is no longer visible is taken as a measure of the transparency of the water. The plain white, 30 cm diameter Secchi disk remains the standard design used in marine studies.

Shellfish: it is a term used in fisheries that includes exoskeleton-bearing aquatic invertebrates used as food such as molluscs, crustaceans, and echinoderms.

Trophozooite: is the activated, feeding stage in the life cycle of certain protozoa.

Chapter 1. General introduction.

Aquaculture production has been increasing in the last decades to meet the increasing global demand for seafood. The forecast of global aquaculture fish production for the year 2023 is estimated in 96 million tonnes of live weight while the total capture of fish is estimated in 89.5 million of tonnes (FAO, 2023). The global meat production forecast for the year 2023 is 364 million tonnes of carcass weight equivalent, it includes bovine, poultry, pig and ovine meat. Globally, aquatic foods provide about 17 percent of animal protein, reaching over 50 percent in several countries in Asia and Africa (FAO, 2022a). Fisheries production systems are under considerable stress from overfishing, habitat loss, pollution, invasive species, water abstraction, damming, and disruption of fisheries by climate change (Allison et al., 2009). Marine aquaculture is seen as a key sector to improve global food and nutrition security due to its great potential for reducing food system environmental stressors (Gephart et al., 2021). Among the different marine aquaculture products, bivalves could contribute 43 percent of aquatic food by 2050 (Costello et al., 2020). The General Fisheries Commission for the Mediterranean (GFCM) of the Food and Agriculture Organization of the United Nations (FAO) designed a strategy to achieve the sustainable management of marine resources in the Mediterranean and the Black Sea (FAO, 2021a). One of the targets of this strategy, target 3, aims at the sustainable development of aquaculture and its contribution to sustainable food systems, working towards the resilience of the sector against global challenges such as climate change and pollution. One of the expected outcomes of the FAO Blue Transformation roadmap is to reach at least 35 percent growth in global sustainable aquaculture production by 2030 (FAO, 2022b). To achieve these objectives, it is necessary to increase the production of marine bivalves. Marine bivalves are low trophic species, and their production system can be classified as non-fed aquaculture. The production of bivalves is frequently referred as shellfish aquaculture which includes other molluscs, crustaceans, and echinoderms. Bivalve aquaculture is currently facing challenges related to: 1) the need for expansion to new coastal zones resilient to climate change (Filgueira et al., 2016); 2) the increase in mortalities associated to new pathogens (Elston et al., 2008) and marine heat waves (Lacoue-Labarthe et al., 2016); 3) chemical (Saldaña-Serrano et al., 2022; Squadrone et al., 2016) and microbial pollution (Hunt et al., 2023; Rowan, 2023) of the coastal waters; 4) harmful algal blooms (Brown et al., 2020; Gianella et al., 2021; Rolton et al., 2022; Stoner et al., 2023; Tan et al., 2023); 5) the difficulties in obtaining local seed (Avdelas et al., 2021); 6) the translocation of invasive species (Cahill et al., 2022; Lacoste and Gaertner-Mazouni, 2015). Scientific research needs to focus on these challenges to help the aquaculture industry to achieve the objectives of increasing bivalve production.

1. Aquaculture production.

1.1. Global aquaculture.

World aquaculture production in 2019 consisted of 56.3 million tonnes of finfish, 17.6 million tonnes of molluscs, 10.5 million tonnes of crustaceans, 977 thousand tonnes of other aquatic animal species and 34 million tonnes of seaweeds (Figure 1- 1). Finfish production from inland aquaculture represented 56.7 percent of world total aquaculture production of aquatic animals in 2019. The annual growth rate for the global fish and shellfish aquaculture production was on average 3.7 percent during 2016–2019. The contribution of aquaculture to the total production of aquatic animals from capture and aquaculture combined has risen steadily from 39.9 percent in 2010 to 48.0 percent in 2019. Preliminary estimates from FAO for 2019 indicate a growth in per capita consumption of fish to about 20.5 kg, with the share of aquaculture production in total available food fish supply overtaking that of capture fisheries (11.1 kg vs 9.5 kg). In 2019[1], world aquatic production from aquaculture was 120 million tonnes while capture production was 93 million tonnes (Figure 1- 2). This comparison may be biased due to the differences in the proportions between capture and aquaculture for the different groups of species. The edible content of molluscs in low in comparison to finfish and the amount of capture production of fish (freshwater, diadromous and marine fishes) was 79 million tonnes and 56 million tonnes were produced by the aquaculture industry. For the rest of species (crustaceans, molluscs, aquatic plants and others) the total amount produced by aquaculture was much higher than the captured production, 64 and 14 million tonnes respectively (FAO, 2021b).

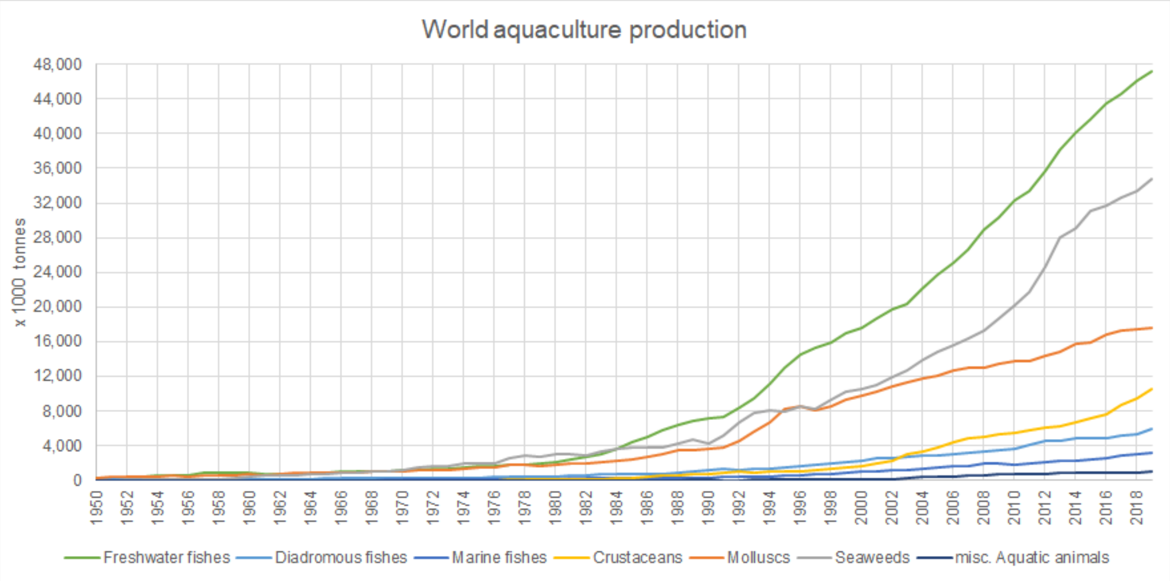

Figure 1- 1. World aquaculture production of fish, crustaceans, molluscs and seaweeds (FAO, 2021b).

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[1] The FAO Fishery and Aquaculture Statistics – Yearbook 2020 was published at the end of September 2023, after the completion of this manuscript. In the year 2020, world aquatic production from aquaculture was 123 million tonnes while capture production was 91 million tonnes. FAO. 2023.Fishery and Aquaculture Statistics – Yearbook 2020. FAO Yearbook of Fishery and Aquaculture Statistics. Rome.

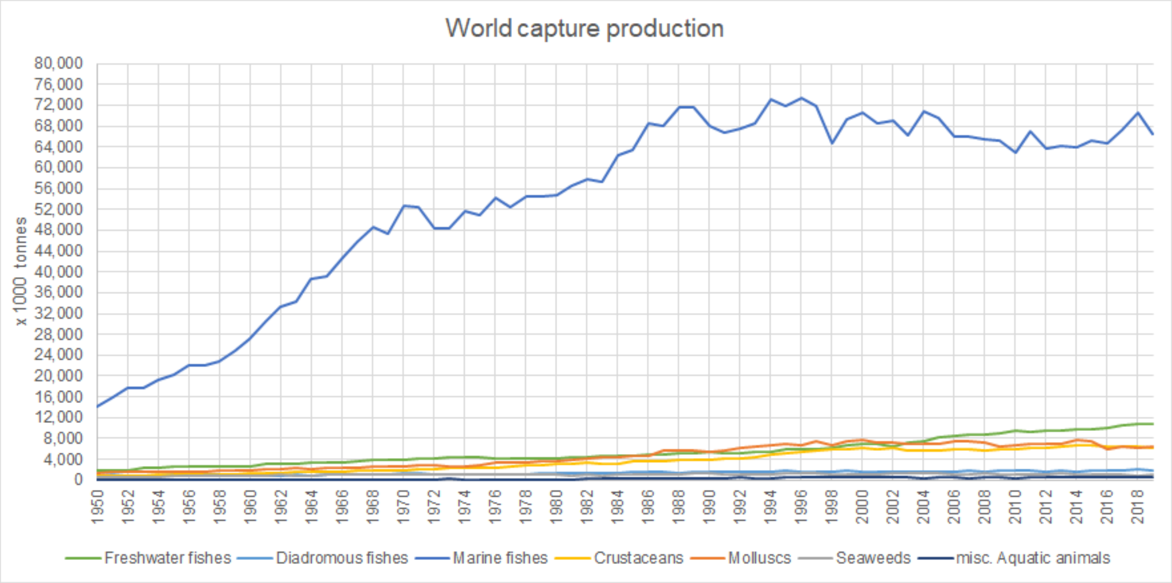

Figure 1- 2. World aquatic production by capture (FAO, 2021b).

1.2. Aquaculture production of bivalves in the Mediterranean Sea.

In the Mediterranean Sea, the countries that have reported aquaculture production of bivalves during 2010-2019 are Albania, Algeria, Bosnia, Croatia, France, Greece, Italy, Montenegro, Morocco, Slovenia, Spain, Tunisia, and Turkey (FAO, 2021b). The species produced are *Mytilus galloprovincialis* Lamarck, *Crassostrea gigas* Thunberg, *Ostrea edulis* Linnaeus, *Ruditapes decussatus* Linnaeus, and *Ruditapes philippinarum* Adams & Reeve (FAO, 2021b). The total production in the Mediterranean Sea during the year 2019, in thousands of tonnes was 105 for *M. galloprovincialis*, 31 for *R. philippinarum* and 5.7 for *C. gigas* (Figure 1- 3). The production of *O. edulis* and *R. decussatus* was 93 and 73 tonnes respectively (FAO, 2021b). For Spain and France, we include only the production from the regions bordering the Mediterranean Sea excluding the production from the Atlantic regions (Agreste, 2021; JACUMAR, 2023). Among the different Mediterranean countries, it is Italy the major producer of farmed bivalves. During the year 2020, Italy produced 50 thousand tonnes of *M. galloprovincialis*, 24 thousand tonnes of *R. philippinarum* and 182 tonnes of *C. gigas*. (Ispra, 2022). Bivalve production in Italy occurs mainly in Emilia Romagna (16 thousand tonnes) and Veneto (32 thousand tonnes), smaller productions are localized in Friuli Venezia Giulia, Liguria, Marche. Lazio, Abruzzo, Molise, Campania, Puglia, Calabria, Sicilia and Sardegna (Ispra, 2022). Bivalve production in Italy which is higher than finfish production, has decreased in Italy during the last 15 years (Ispra, 2022).

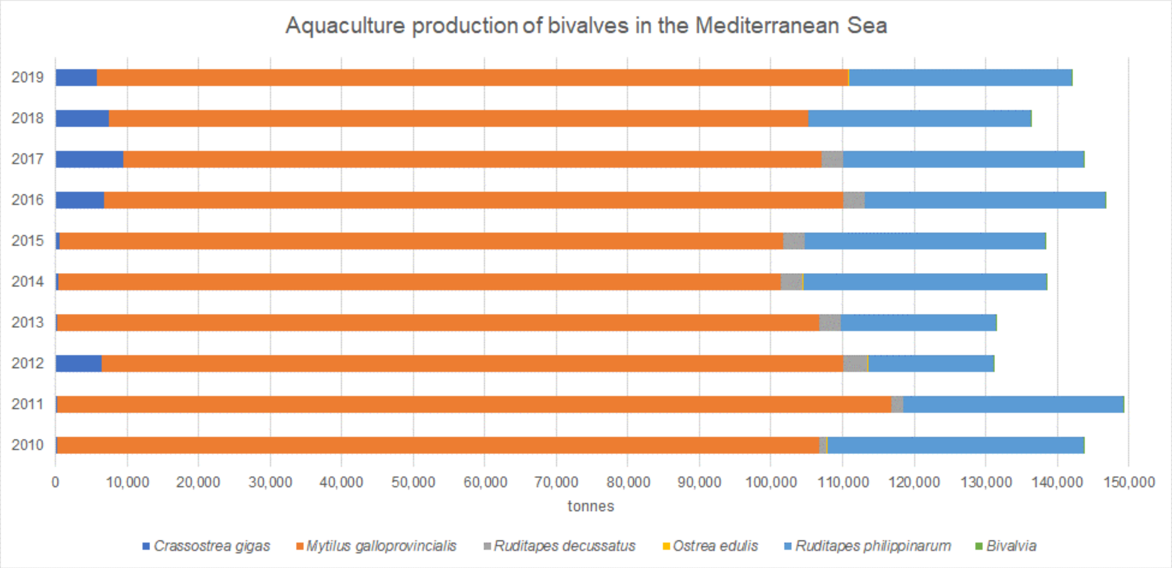

Figure 1- 3. Aquaculture production of bivalves in the Mediterranean Sea (FAO, 2021b).

Italy produces 59 percent of the total Mediterranean aquaculture production of *M. galloprovincialis* (Figure 1- 4) followed by Greece (22 percent), France (6 percent), Spain (5 percent) and Turkey (4 percent).

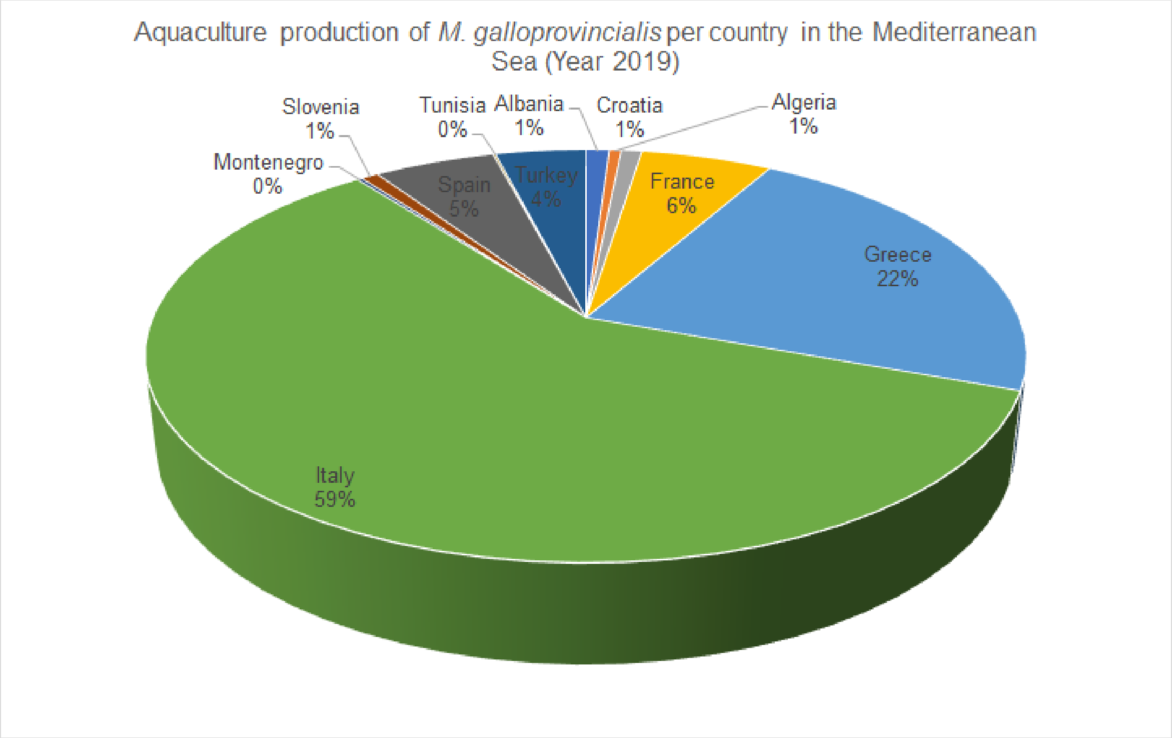

Figure 1- 4. Aquaculture production of *M. galloprovincialis* in the Mediterranean Sea (FAO, 2021b).

There is a high seasonal demand of mussels in the Mediterranean countries due to tourism. Local aquaculture production is not sufficient. In spite of the high demand, mussel aquaculture in the Mediterranean has not increased in the last years in contrast to the increase of the global production. Research for the identification of suitable farming areas is needed in some countries (Avdelas et al., 2021).

1.3. Marine Aquaculture in Spain.

In the year 2022, Spain produced 59 thousand tonnes of finfish for a value of 541 million Euros, 378 tonnes of crustaceans (2,6 million Euros), 227 thousand tonnes of bivalve molluscs (176 million Euros), 2 tonnes of microalgae (1,7 million Euros) and 19 tonnes of seaweed (152 thousand Euros) (Figure 1- 5, Figure 1- 6, Figure 1S- 1, Figure 1S- 2, Figure 1S- 3). The species produced for each of these groups are the following (JACUMAR, 2023);

Finfish: *Sparus aurata* Linnaeus; *Solea senegalensis* Kaup; *Dicentrarchus labrax* Linnaeus; *Mugil* Linnaeus; *Chelon labrosus* Risso; *Seriola dumerili* Risso; *Scophthalmus maximus* Linnaeus; *Diplodus sargus* Linnaeus; *Argyrosomus regius* Asso; *Dicentrarchus punctatus* Bloch; *Thunnus thynnus* Linnaeus; *Anguilla anguilla* Linnaeus;

Crustaceans: *Penaeus japonicus* Spence Bate; *Penaeus vannamei* Boone; *Penaeus kerathurus* Forskål; *Palaemon varians* Leach;

Bivalve molluscs: *Aequipecten opercularis* Linnaeus; *Ostrea edulis* Linnaeus; *Crassostrea gigas* Thunberg; *Ensis* Schumacher; *Mytilus galloprovincialis* Lamarck; *Venus verrucosa* Linnaeus; *Cerastoderma edule* Linnaeus; *Ruditapes philippinarum* A. Adams & Reeve; *Ruditapes decussatus* Linnaeus; *Venerupis corrugata* Gmelin;

Microalgae: *Dunaliella salina* (Dunal) Teodoresco; *Tetraselmis* F.Stein; *Tetraselmis chui* Butcher; *Microchloropsis gaditana* (L.M.Lubián) M.W.Fawley, I.Jameson & K.P.Fawley;

Seaweed: *Ulva lactuca* Linnaeus; *Ulva rigida* C. Agardh; *Gracilaria* Greville; *Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders; *Codium tomentosum* Stackhouse; *Codium vermilara* (Olivi) Delle Chiaje; *Gracilaria dura* (C. Agardh) J. Agardh; *Gracilariopsis longissima* (S.G. Gmelin) Steentoft, L.M. Irvine & Farnham;

Some species are produced in both, the Mediterranean and the Atlantic coasts of Spain but from the species listed above, the following species are not produced in the regions bordering the Mediterranean coast of Spain or at least not in a relevant amount: *Penaeus vannamei*, *Dunaliella salina*, *Tetraselmis chui*, *Saccharina latissima*, *Ulva rigida*, *Codium tomentosum*, *Codium vermilara*, *Gracilaria dura*, *Gracilariopsis longissima*, *Scophthalmus maximus*, *Aequipecten opercularis*, *Ostrea edulis*, *Cerastoderma edule*, *Venerupis corrugata* (Figure 1- 5, Figure 1- 7, Figure 1- 8).

Catalonia reports the production of razor clams as produced in aquaculture, since we are aware that this production has been captured by shellfish harvesters, we have excluded this production from the total Spanish production on bivalve aquaculture (2010-2022).

Most of the aquaculture production of crustaceans in Spain is located in Andalusia (370 tonnes, 2.6 million euros), as well as the production of microalgae (1.57 tonnes, 1.6 million Euros), Andalusia produces 7 tonnes of seaweed (*Ulva lactuca* and *Gracilaria*) for a value of 118 thousand Euros. The production of marine finfish in Spain is mainly located in the Mediterranean coast (43 thousand tonnes, 405 million Euros). Among the different species, the production of *Sparus aurata* and *Dicentrarchus labrax* is mainly located along the Mediterranean coast, in the regions of Andalusia, Murcia, Valencia and Catalonia. There is a small production of *Solea senegalensis* (363 tonnes), *Mugil* and *Chelon labrosus* (45 tonnes), *Diplodus sargus* (1 tonnes) and *Dicentrarchus punctatus* (0.38 tonnes) in Andalusia and a small production (147 tonnes) of *Seriola dumerili* in Murcia and Valencia. *Argyrosomus regius* (4.7 tonnes) is produced in Andalusia, Murcia and Valencia. The whole Spanish production of *Thunnus thynnus* is located in Murcia (8 thousand tonnes) and Catalonia (2. 6 thousand tonnes). There is a small production of *Anguilla anguilla* (335 tonnes) in Valencia. The production of bivalves in Spain is mainly located in the Atlantic coast (222 thousand tonnes), only 6 thousand tonnes are produced in the Mediterranean coast for a value of 10 million Euros. Along the Mediterranean coast, *Crassotrea gigas* is produced in Andalusia (28 tonnes), Valencia (42 tonnes) and Catalonia (397 tonnes); *Ruditapes philippinarum* is produced in Andalusia (4.67 tonnes) and Catalonia (6 tonnes) (Figure 1- 8); *Mytilus galloprovincialis* is produced in the Balearic Islands (104 tonnes), Andalusia (640 tonnes), Valencia (1.8 thousand tonnes), Catalonia (2.9 thousand tonnes) (Figure 1- 9).

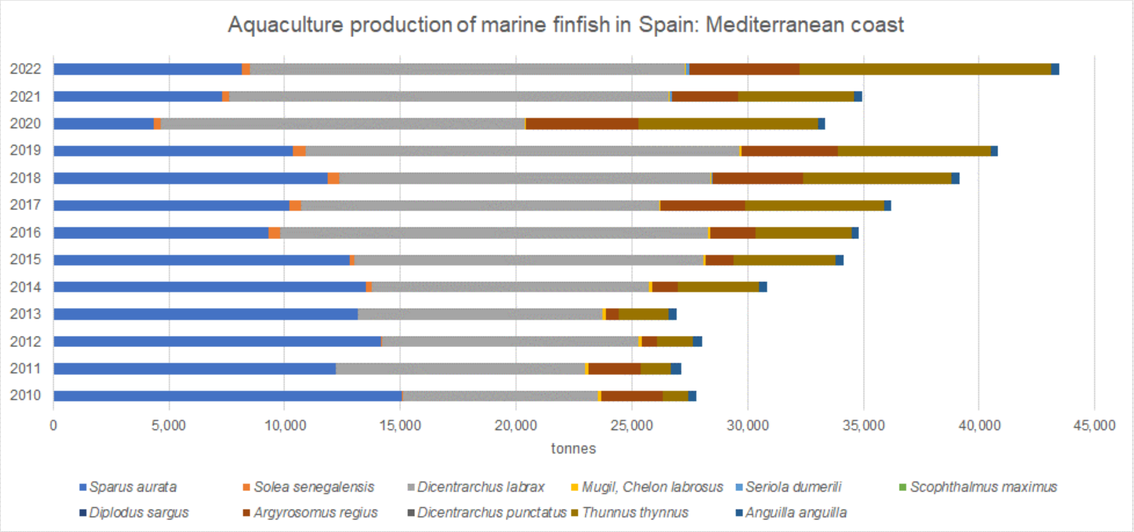

Figure 1- 5. Aquaculture production of marine finfish in the Mediterranean coast of Spain (JACUMAR, 2023).

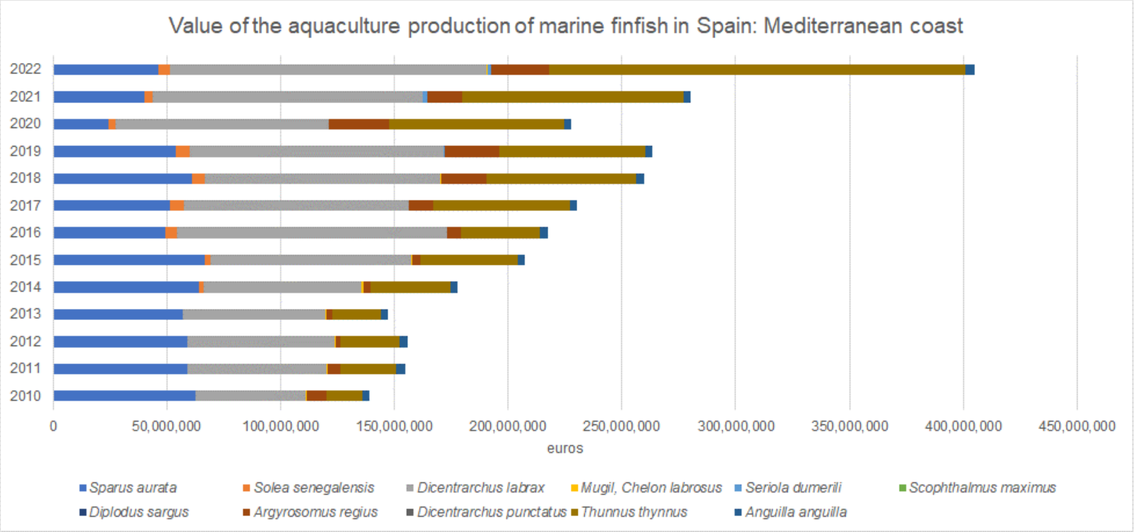

Figure 1- 6. Value of the aquaculture production of marine finfish in the Mediterranean coast of Spain (JACUMAR, 2023).

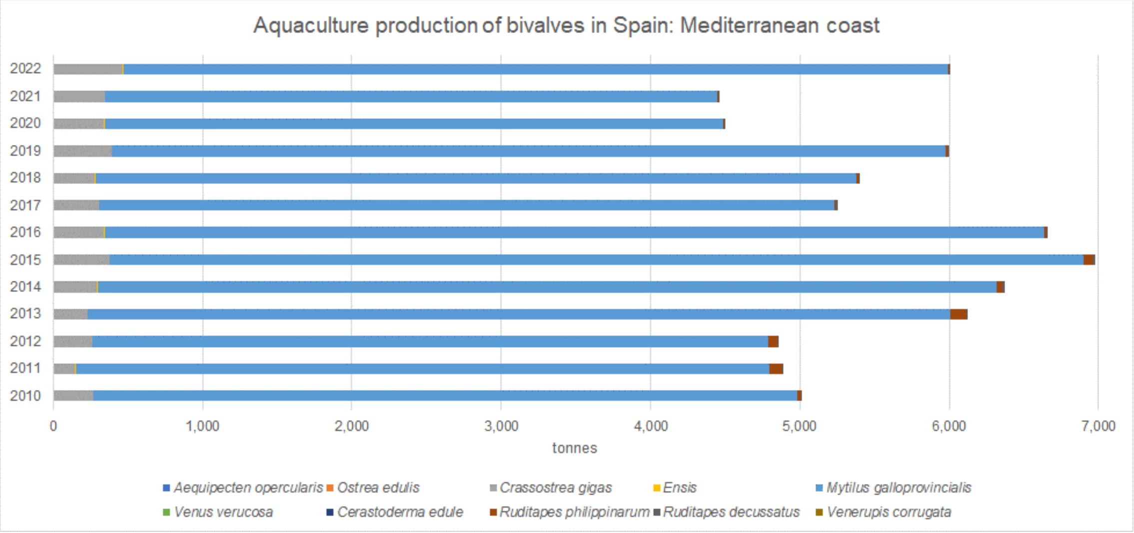

Figure 1- 7. Aquaculture production of bivalve molluscs in the Mediterranean coast of Spain (JACUMAR, 2023).

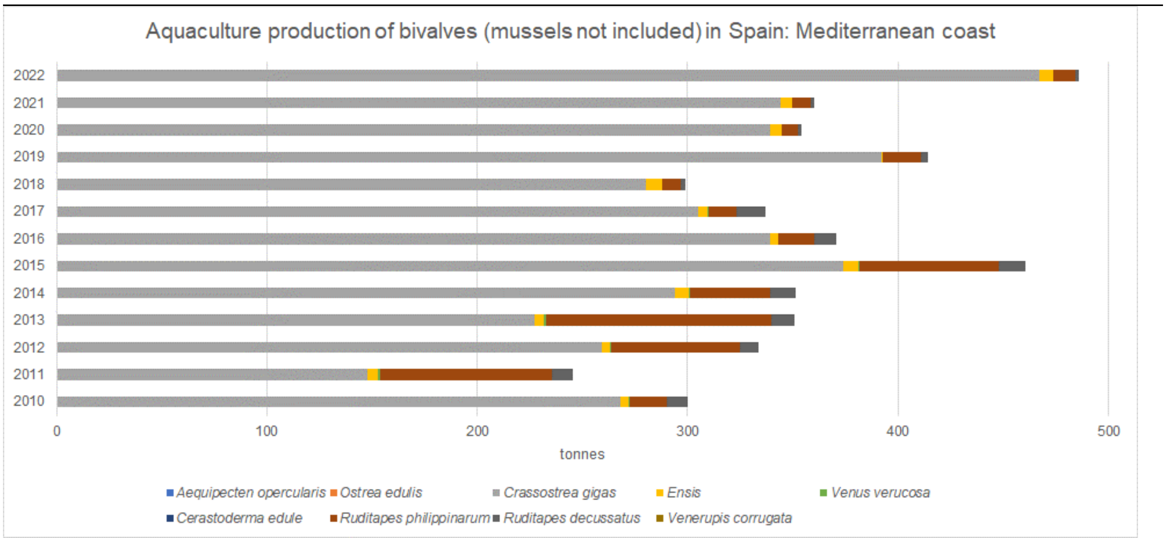

Figure 1- 8. Aquaculture production of bivalves, excluding mussels, in the Mediterranean coast of Spain (JACUMAR, 2023).

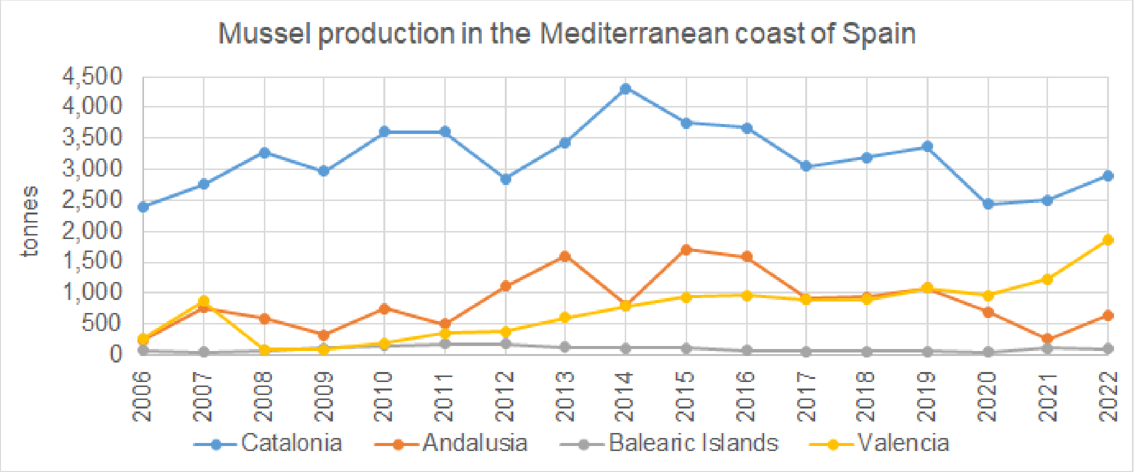

Figure 1- 9. Mussel production in the different regions of the Mediterranean coast of Spain (JACUMAR, 2023).

2. The Mediterranean Sea

The Mediterranean Sea is connected with the North Atlantic Ocean through the Strait of Gibraltar, with the Black Sea through the Turkish Strait System (Dardanelles, Marmara Sea and Bosphorus Strait). It is a concentration basin where evaporation exceeds precipitation. The surface Atlantic water entering through the Strait of Gibraltar circulates along the North African coast, the main part flows through the Sardinia Channel into the Tyrrhenian Sea and a smaller part flows through the Sicily Channel into the eastern Mediterranean Sea. In the northern Tyrrhenian Sea one branch flows through the Corsica channel. The principal flow in the eastern Mediterranean is in a cyclonic circulation but in the northern Ionian Sea, the North Ionian Gyre shifts between cyclonic and anti-cyclonic on decadal time scales. The surface Atlantic Water is transformed in Levantine Intermediate Water in the area east of Rhodes and in the Cretan Sea and from there it spreads to the Levantine basin and to the Ionian Sea, it passes the Sicily channel, tourns around the Tyrrenian Sea and enters the western Mediterranean through the Sardinia Channel, a fraction flows out the Tyrrhenian Sea northwards entering the Ligurian Sea and the Provençal Basin (Tanhua et al., 2013). Mesoscale processes play a key role in determining the large-scale circulation, the distribution of water masses and their mixing, meanders, eddies, and filaments mainly originate as instabilities of large-scale currents and fronts (Mason et al., 2023). Salinity ranges from 36.1 in the Atlantic water at the entrance in the Strait of Gibraltar, to 39.1 at the surface in the eastern Mediterranean Sea (Schroeder et al., 2023). The influence of the major rivers (Po, Rhone and Nile) can be observed in the Sea Surface Salinity. The Mediterranean Sea is oligotrophic, even ultra-oligotrophic and the ratio N:P is higher than in the world ocean. The deep Mediterranean water exported to the Atlantic Ocean has a higher concentration of nutrients than the Atlantic Surface water entering through the Strait of Gibraltar (Tanhua et al., 2013). The Mediterranean exports inorganic N and P to the Atlantic, and imports organic N and P from the Atlantic, less bioavailable for phytoplankton, mineralization processes are predominant (Álvarez et al., 2023). The Mediterranean Sea is becoming warmer and saltier, future projections predict that SST will continue increasing throughout the basin as well as the surface salinity in the Levantine basin and the Aegean Sea.

3. The Ebro delta as a model.

The Ebro delta concentrates most of the bivalve aquaculture production from the Mediterranean coast of Spain. Mussels (*M. galloprovincialis*) and oysters (*C. gigas*) are grown in ropes hanging from rafts in the two coastal embayments of Alfacs Bay and Fangar Bay (Figure 1- 10). Mussel production for the year 2022 was 2,272 tonnes in Alfacs Bay and 552 tonnes in Fangar Bay. The production of oyster for the same year was 25 tonnes in Alfacs Bay and 372 tonnes in Fangar Bay. In some of the rafts, shellfish farmers hang mussel seed collectors but most of the mussel seed is imported from Italy and Greece. Oyster seed is imported from shellfish hatcheries and natural collection, both from France. In these two coastal embayments there are some areas dedicated to grow clams (*R. philippinarum*) but its production has been highly impacted by the invasion of the blue crab (*Callinectes sapidus* Rathbun). There is also mussel aquaculture outside these two coastal bays. A long-line is installed in the south of the Ebro delta (80 tonnes/year 2022) and there is high interest in installing new farms in the northern area outside Fangar Bay.

Figure 1- 10. Maps showing the location of the Ebro delta in the Western Mediterranean and the bivalve farms in Alfacs Bay and Fangar Bay, the red rectangles show the rafts where shellfish farmers grow oysters and mussels.

Massive mussel mortalities occur in summer when seawater temperature rises above 28 °C (Ramon et al., 2007). The first known mussel mortality event that occurred in the Ebro delta associated to high seawater temperature happened in 1994 in Alfacs Bay, the second occurred in the year 2003 and affected both bays. These events of high seawater temperature in summer, are increasing in frequency and duration (Figure 1- 11). Shellfish farmers have adapted the calendar of seeding and harvesting with the objective of harvesting the whole production before seawater temperature in the two coastal embayments reaches 28 °C. The mussel seed collected within the embayments at the beginning of each year will reach commercial size 12-18 months later, the mussel seed collected and grown in the two embayments does not survive in years when seawater temperature rises above 28 °C during more than one week. Some farmers prefer to dedicate the whole farm to grow imported seed since they face the risk of not only a losing the production of the year but also the production of the year after. Marine heat wave events are also increasing in their geographical extent and therefore other Mediterranean coastal areas could be affected at the same time hampering the obtention of mussel seed for starting new cycles of production. Some shellfish farmers requested to open a channel at the interior of Alfacs Bay to connect the inner part of the Bay with the open sea. The results of the simulations showed that the integrated values of seawater temperature over the bay did not show significant variations between the tests and the control case, with differences smaller than 0.07 °C (Cerralbo et al., 2019). Finding new appropriate locations to maintain the seed and to grow mussels up to commercial size during summer is a requirement not only to increase mussel production but also to maintain the current production in the future years.

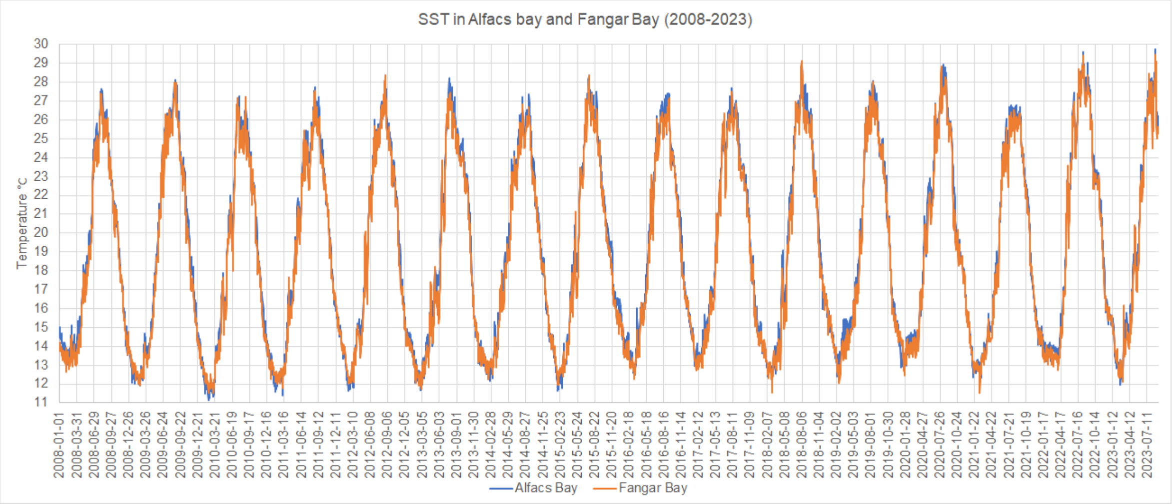

Figure 1- 11. SST at the coastal embayments of Alfacs Bay and Fangar Bay measured by remote sensing using the Mediterranean Sea High Resolution and Ultra High-Resolution Sea Surface Temperature Analysis. Product ID: SST_MED_SST_L4_NRT_OBSERVATIONS_010_004 from Copernicus. https://doi.org/10.48670/moi-00172.

Events of oyster seed mortality were frequent during the first decade of 2000s in the Ebro delta, and critical between 2008-2014, these events were associated to the virus OsHV-1 microvar (Roque et al., 2012). The studies showed that there was a range of critical seawater temperature that induced the infection (Delisle et al., 2020; Delisle et al., 2018; Garcia et al., 2011). A strategy based on avoiding the seeding of new spat during the critical time of the year based on seawater temperature has reduced these mortality events (Carrasco et al., 2017). The parasite *Marteilia* Grizel, Comps, Bonami, Cousserans, Duthoit & Le Pennec, has been detected in mussels from the Ebro delta (Carrasco et al., 2008) but not associated to mussel mortalities. Massive mussel mortality events are scarce (Lupo et al., 2021) and this scarcity in massive mortalities has been linked to the presence of a high number of sequences related to immune processes and killing molecules in *M. galloprovincialis* in comparison to other bivalve species (Moreira et al., 2018). Mussel mortalities in the Ebro delta have been related to blooms of ichthyotoxic dinoflagellates and to marine heat waves (Figure 1- 12), but recently unexplained mortalities have been registered in some farms located in Alfacs Bay.

Figure 1- 12. Mussel farms in Alfacs Bay. A) Hanging ropes growing mussels. B) Raft dedicated to collection of mussel seed. C) Mussel mortality event during the marine heat wave in summer 2003.

Invasive species are also a threat for bivalve aquaculture in the Ebro delta, the colonial tunicate *Didemnum vexillum* Kott grows on the shell of oysters and farmers need to frequently clean the shells to avoid suffocation (Ordonez et al., 2015). Another invasive species, *Clavelina oblonga* Herdman, affects mainly mussel aquaculture growing on top of mussel ropes during summer (Ordonez et al., 2016). The seasonal patterns of ascidians in the Ebro delta have been studied and the results recommend the preference for spat immersion during fall and below 1 m depth to reduce fouling on bivalves (Casso et al., 2018).

The translocation of animals between different aquaculture areas represents a high risk of introducing invasive species and pathogens (Brenner et al., 2014). The use of local seed has as well other advantages since an adaptation to local conditions has been observed in some species (Masanja et al., 2023; Palumbi et al., 2019; Thomsen et al., 2017).

Production of local bivalve spat in hatcheries requires the production of large quantities of cultured microalgae which may be a limiting factor. The use of commercial phytoplankton concentrates may improve the efficiency of bivalve hatcheries reducing the investment and labour costs. There aren't too many reports on the use of these concentrates for bivalve aquaculture and the results are often contradictory (Hassan et al., 2021; Supono et al., 2023).

Harmful algal blooms (HABs) have been a threat for bivalve aquaculture in the Ebro delta due to the ichthyotoxic dinoflagellates *Karlodinium veneficum* (D. Ballantine) J. Larsen and *Karlodinium armiger* Bergholtz, Daugbjerg & Moestrup that produced massive mortalities of marine fauna between 1994 and 2000 (Bergholtz et al., 2006; Delgado and Alcaraz, 1999; Fernandez-Tejedor et al., 2004; Fernandez-Tejedor et al., 2007; Garces et al., 1999; Garces et al., 2006; Guallar et al., 2016; Krock et al., 2017). Since the year 2000, blooms of these ichthyotoxic species have reduced its frequency and intensity. Other HAB species have also been a threat for bivalve aquaculture in the area since the first event occurred in 1989 associated to a bloom of *Alexandrium minutum* Halim (Delgado et al., 1990). Closures due to the detection of Diarrhetic Shellfish Poisoning (DSP) over regulatory levels were frequent during the first decade of 2000s associated to blooms of *Dinophysis* *sacculus* F. Stein (García-Altares et al., 2016). At a global level, it has been suggested that the intensified monitoring efforts associated with increased aquaculture production are responsible for the perceived increase in harmful algae events and that there is no empirical support for broad statements regarding increasing global trends. Instead, trends need to be considered regionally and at the species level (Hallegraeff et al., 2021). HABs trends in the Ebro delta were assessed in 2010 and a huge increase in blooms of *Pseudo-nitzschia* species was observed (Fernández-Tejedor et al., 2010) but not associated to production of domoic acid over regulatory levels (Gimenez Papiol et al., 2013).

The concentration of different pesticides in water and sediments of the Ebro delta represents a hazard for aquatic organisms (Garcia-Pimentel et al., 2023; Peris et al., 2022) specially during the rice growing season (Vittoria Barbieri et al., 2021). Moreau et al. (2015) suggested that pesticides may be an additional stress factors that render oysters more susceptible to pathogens triggering massive mortality outbreaks. Some pesticides induce alterations on the bivalve's immune system (Gagnaire et al., 2006). Sublethal interactive effects of different pesticides have been observed on bivalve growth and condition index (Tissot et al., 2022). There are methodological limitations for a comprehensive monitoring of all the spectrum of possible present chemical contaminants due to the high variety and frequent changes in the products used (Alvarez-Munoz et al., 2015a; Alvarez-Munoz et al., 2018; Alvarez-Munoz et al., 2015b; Alves et al., 2017; Alves et al., 2018; Aznar-Alemany et al., 2017; Barbosa et al., 2018; Cunha et al., 2015; Cunha et al., 2018; Maulvault et al., 2015). Mixtures of different pesticides are found in the aquatic environment, experimental approaches should include testing the acute and sublethal effects of these mixtures in bivalve adults and juveniles (Renault, 2011).

4. Thesis objectives.

The development of low trophic aquaculture has become a priority at global level to reduce the gap between demand and supply of seafood products (Krause et al., 2022). Low trophic aquaculture includes the cultivation of primary producers such as micro- and macroalgae and primary consumers such as bivalves and herbivore fish (Slater and James, 2023). In some countries such as Spain and France, bivalve production accounts for a high percentage of total animal aquaculture, being 74.8 % and 75.4 % respectively (FAO, 2022a). Bivalve aquaculture provides environmental benefits such as nutrient remediation (van der Schatte Olivier et al., 2020) and increase of biodiversity (Maurin et al., 2019) in addition to the value of food production. The progress and expansion of shellfish aquaculture relies in defining the right areas for production and in the implementation of monitoring programs that address biosecurity and food safety. The use of remote sensing for aquaculture site selection has been proposed in different geographical areas (Brigolin et al., 2015; Liu et al., 2014; Newell et al., 2021; Snyder et al., 2017) but requires the development of local appropriate algorithms for shallow coastal areas.

The hazards for shellfish aquaculture are related to the decrease or loss of production as well as to the loss of hygienic conditions of the final product for human consumption. The first category is related to seafood security, this category includes: ocean warming, acidification, reduced access to spat, pathogens, invasive species, lack of suitable coastal areas. The second category is related to seafood safety, and it includes blooms of toxic phytoplankton and their toxins, microbial and chemical contamination. Global change has important impacts on all these aspects and adds uncertainties to the future of shellfish aquaculture (Naylor et al., 2021).

Since 1987, there has been a substantial and significant increase in the frequency of marine heat waves in the Mediterranean Sea (Dayan et al., 2023). Marine heat waves are associated with the onset of mass mortality events of marine organisms in the Mediterranean Sea (Garrabou et al., 2022). Heat stress on bivalves has an impact on cellular damage and oxidative stress, leading to impaired immune function, elevated inflammation, shifts in microbial populations, and reduced growth (Masanja et al., 2023). Therefore, it is crucial to locate shellfish farms in areas less impacted by marine heat waves. Temperature also shapes the structure and distribution of phytoplankton communities which are the primary food for filter-feeding bivalves. Some phytoplankton species produce toxins that have an effect in seafood safety and security. Ocean warming has significantly increased the potential mean growth rate and duration of bloom season for some toxic species in the North Atlantic and the North Pacific Oceans (Gobler et al., 2017). An increase in HABs events could be limiting for bivalve aquaculture. Consequently, it is necessary to assess the trends and expansion of HABs in Mediterranean waters.

The coastal embayments of the Ebro delta concentrate most of the bivalve production of the Mediterranean coast of Spain. Massive mussel mortality events occur in these areas during marine heat wave events (Ramon et al., 2007). Mussel populations do not seem to be affected by pathogens in the same extent as oysters and clams (Rey-Campos et al., 2019; Romero et al., 2014; Venier et al., 2011). For this reason, it is essential to describe the absence or presence of mussel pathogens during mortality events not associated to marine heat waves.

The impacts of invasive species in bivalve aquaculture of the Ebro delta have been addressed and strategies to reduce these impacts have been designed (Casso et al., 2018; Ordonez et al., 2015; Ordonez et al., 2016).

Mortalities of oyster seed in the Ebro delta has been assessed and recommendations have been issued (Carrasco et al., 2017). These recommendations are applied by the farmers and have reduced mortalities of oyster seed. There is always a risk of introducing new pathogens when bivalves are translocated from or to different geographical areas. The application of new methods to obtain local oyster seed is a must that would benefit the aquaculture industry. A limiting step for it is the production of live phytoplankton to feed the stocks. The use of commercial phytoplankton concentrates could facilitate the production of local seed by the shellfish farmers (Willer and Aldridge, 2017; Willer and Aldridge, 2019). Then, it is required to test the performance of commercial phytoplankton concentrates to produce local oyster seed.

4.1. Hypothesis.

1) Remote sensing imagery in shallow coastal areas has a low performance in comparison to oceanic waters, the development of algorithms appropriate for these areas will allow to use this valuable information to find suitable areas for bivalve aquaculture.

2) Harmful algal blooms are a threat for bivalve aquaculture in many parts of the world, and an increase in the number of blooms has been observed in some geographical areas. Creating a database based on information retrieved from publications in combination with information from open access databases will allow to evaluate the risks of harmful algal blooms in the Mediterranean Sea and their trends.

3) The increase of mussel mortalities observed may be related to the presence of parasites that may be new or previously reported as pathogens in other bivalve species.

4) Concentrated based products used instead of live phytoplankton to feed bivalve seed in the hatchery and nursery phases may facilitate production of local seed.

4.3. Specific objectives.

Objective 1) To develop a methodology to provide an accurate estimation of chlorophyll a concentration in coastal areas of the Ebro delta and use it to rank the suitability and the carrying capacity of these areas for mussel aquaculture.

Objective 2) To assess the trends and expansion of harmful algal blooms in the Mediterranean Sea.

Objective 3) To describe the presence or absence of parasites that could be involved in mussel mortalities occurred in the Ebro delta.

Objective 4) To assess if phytoplankton concentrated based products may be used to produce local bivalve seed at low cost.

4.4. Long-term objective.

To assess the challenges for bivalve aquaculture in the Ebro delta in the context of global change and provide some tools to facilitate its development contributing to the success of bivalve aquaculture in the Mediterranean Sea.

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Chapter 2. New shellfish growing areas in open waters.

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Title: Accurate Estimation of Chlorophyll-a Concentration in the Coastal Areas of the Ebro Delta (NW Mediterranean) Using Sentinel-2 and Its Application in the Selection of Areas for Mussel Aquaculture.

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Abstract

Multispectral satellite remote sensing imagery, together with appropriate modeling, have been proven to provide chlorophyll-a maps that are useful to evaluate the suitability of coastal areas for carrying out shellfish aquaculture. However, current approaches used for chlorophyll-a estimation in very shallow coastal areas often fail in their accuracy. To overcome this limitation, an algorithm that provides an accurate estimation of chlorophyll-a concentration in the coastal areas of the Ebro delta (North Western Mediterranean) using atmospherically corrected Sentinel 2 (S2) remote sensing reflectances (Rrs) has been calibrated and validated. The derived chlorophyll-a maps created have been used in a dynamic carrying capacity model that covers areas from very rich waters inside the embayment to the more oligotrophic waters in the open sea. The use of carrying capacity models is recommended to evaluate the potential of marine coastal areas for bivalve mollusk aquaculture. In this context, the depletion of chlorophyll-a is an indicator of negative environmental impact and thus a continuous monitoring of chlorophyll-a is key. The proposed methodology allows estimation of chlorophyll-a concentration from Sentinel-2 with an accuracy higher than 70% in most cases. The carrying capacity and the suitability of the external areas of the Ebro delta have been determined. The results show that these areas can hold a significant mussel production. The methodology presented in this study aims to provide a tool to the shellfish aquaculture industry.

Keywords:

Sentinel-2; aquaculture; carrying capacity; chlorophyll-a; shellfish; bivalves; water quality

1. Introduction

Remote sensing enables the evaluation of the spatial and temporal variability of water quality worldwide, overcoming the lack of data from new, remote, or large marine areas. This has led to several applications of remote sensing in bivalve mollusk aquaculture, including spatial planning (Brigolin et al., 2017; Giordano et al., 2019; Habbane et al., 1997; Hou et al., 2022; Radiarta and Saitoh, 2009), early detection of harmful algal blooms (Babin et al., 2008; Silva et al., 2016; Torres-Palenzuela et al., 2019; Touza et al., 2004; Wakamatsu et al., 2022; Wolny et al., 2020), and detection of microbial contamination (DeLuca et al., 2020; Uz et al., 2020). Among the different water quality parameters of interest that can be measured by sensors from satellites, chlorophyll-a concentration is commonly used in bivalve mollusk aquaculture because it is considered the best proxy of phytoplankton biomass (Huot et al., 2007), which is one of the ecosystem component Essential Ocean Variables (EOVs) (Miloslavich et al., 2018; Muller-Karger et al., 2018). The retrieval of water quality parameters (such as chlorophyll-a) using satellite remote sensing imagery and appropriate modelling is still challenging in coastal waters, especially in case-2 waters (Pahlevan et al., 2021; Staehr et al., 2022). Although many approaches have been proposed, there is not yet any standardized approach. In case-2 waters, inorganic and/or organic sediments represent an important or dominant contribution to the optical properties (Gordon and Morel, 1983), requiring high accuracy and precision in the atmospheric correction algorithms to successfully retrieve water constituents. Recently, several advances concerning enhanced modelling have been proposed to solve atmospheric correction (Soriano-Gonzalez et al., 2022), but their performances differ depending on the scenario (sun and observation geometry, atmospheric, optical) and site-specific conditions. The lack of a common approach and performance makes it necessary to continue validating different atmospheric correction approaches as well as water quality retrieval methods with in situ data accounting for a wide variety of water types and environmental conditions. Therefore, the estimation of chlorophyll-a concentration in coastal waters still has some difficulties in comparison to oceanic waters due to the more complex optical properties and to high spatial variability. Atmospheric correction and the correction of scale effects are necessary to accurately estimate chlorophyll-a concentration in coastal waters (Chen et al., 2013), where the retrieval of chlorophyll-a concentration through remote sensing has many applications, such as the evaluation of the environmental status of these water masses (Staehr et al., 2022) or spatial planning for bivalve aquaculture (Brigolin et al., 2017).

Global aquaculture production in 2020 reached 17.7 million tons of mollusks, with bivalve aquaculture accounting for a high percentage of total aquaculture production in several countries such as New Zealand, France, Spain, republic of Korea, Italy, and Japan (FAO, 2022a), with China being the main producer (Naylor et al., 2021). Bivalves do not require artificial feeds; they graze on phytoplankton communities present in marine waters (Lucas et al., 2016). Bivalve aquaculture, together with the cultivation of macroalgae and some herbivorous fish species, utilizes low trophic marine species that can provide alternatives for food production with lower environmental impacts (Krause et al., 2022). Bivalve aquaculture is carried out in coastal waters, mainly in estuaries and embayments where phytoplankton biomass is higher than in offshore waters. The global production of bivalves is expected to continue growing (Wijsman et al., 2019); however, seawater temperature in shallow coastal areas is currently reaching thresholds that are limiting the survival of some bivalve species.

Heat waves have been affecting, in recent years, shellfish species in different geographical areas such as the Pacific Northwest in 2021 (Raymond et al., 2022; White et al., 2023), the eastern English channel in 2018 (Seuront et al., 2019), and the coast of California in 2004 (Harley, 2008). In the Mediterranean Sea, the warming trend in Sea Surface Temperature (SST) during the period 1992–2020 was 0.038 +/− 0.002 °C/year (Juza and Tintore, 2021). Marine heat wave (MHW) events in the Mediterranean Sea are increasing in intensity, duration, and frequency (Juza et al., 2022). High seawater temperature in shallow coastal areas during MHW events produces high rates of mortality of *Mytilus galloprovincialis* (Ramon et al., 2007), one of the main species in shellfish aquaculture in the Mediterranean Sea. These issues revealed the need for seeking new shellfish growing areas located in cooler waters, which requires the assessment of the production potential of new culture areas, with a sustainable approach. In this context, the coupling of remote sensing data and carrying capacity models has demonstrated a great potential for monitoring shellfish aquaculture and supporting management strategies (Guyondet et al., 2010; van der Veer and Alunno-Bruscia, 2006).

Carrying capacity models are used to determine the total bivalve biomass supported by a given ecosystem as a function of the water residence time, primary production, and bivalve clearance rate (Dame and Prins, 1997; Smaal and van Duren, 2019). Among them, the Dynamic Energy Budget (DEB) theory based on Kooijman (1986) has been applied to model shellfish growth (Cardoso et al., 2006a; Dong et al., 2022; Fuentes-Santos et al., 2019; Troost et al., 2010) in order to evaluate food limitation for different bivalve species such as *Macoma balthica*, *Mya arenaria*, *Cerastoderma edule*, *Mytilus edulis*, and *Crassostrea gigas* (Cardoso et al., 2006b), and for *Mytilus galloprovincialis* (Duarte et al., 2012), and to predict the impact of environmental changes on shellfish populations (Stechele et al., 2022a). Used alone, or coupled with other biogeochemical (Filgueira et al., 2014), ecophysiological, and box models (Pete et al., 2020), DEB models have been proven to be a valid methodology to determine the quality of an area for shellfish aquaculture (Guyondet et al., 2010; van der Veer and Alunno-Bruscia, 2006). DEB models allow one to calculate the amount of energy used for shellfish growth and reproduction through the ingestion of the available food, thus they benefit from remote sensing estimates (Barille et al., 2020; Gernez et al., 2014; Graham et al., 2020; Palmer et al., 2020; Stechele et al., 2022b).

The Ebro delta, located in the western Mediterranean Sea, is a natural protected area where agriculture, fisheries, aquaculture, and tourism are the main economic activities. The aquaculture industry in the Ebro delta produces 3400 Tn of mussels and 300 Tn of oysters per year (average 2010–2021 (Gencat, 2022)). Shellfish aquaculture is carried out in the two coastal embayments located in the northern (Fangar Bay) and the southern (Alfacs Bay) shores. The hydrodynamics in these embayments is highly sensitive to wind variability, which affects circulation patterns, water exchange times (Cerralbo et al., 2016), and phytoplankton dynamics (Balsells et al., 2021). Drainage channels discharge freshwater from the rice fields into the embayments; the flow of freshwater is variable during the rice growing season from May to September, and it is almost negligible during the rest of the year. Shallowness and large water renewal times, coupled with the exacerbation of MHW events, is limiting the development of aquaculture in these embayments. Previous studies have focused on estimating chlorophyll-a concentration inside the embayments from remote sensing data (i.e., Sentinel-2 MultiSpectral imagery), either for monitoring phytoplankton biomass (Soriano-Gonzalez et al., 2019), or to evaluate the impact of coastal storms on water quality (Angelats et al., 2022). None of them have attempted to model the carrying capacity of the embayments or to evaluate the suitability of the nearby Mediterranean waters for shellfish aquaculture. These are crucial topics for the future of shellfish aquaculture in the region of the Ebro delta and other Mediterranean areas.

This study focuses on the development of a methodology to create, calibrate, and validate an algorithm that provides an accurate estimation of chlorophyll-a concentration in the coastal areas of the Ebro delta (NW Mediterranean) using atmospherically corrected Sentinel 2 (S2) remote sensing reflectances (Rrs) and a tool to calculate the carrying capacity of the potential new aquaculture areas and to evaluate its suitability. For this purpose, the DEB model has been implemented using two-year estimates of chlorophyll-a concentration derived from Sentinel-2 imagery and additional site-specific auxiliary data. The proposed approach is based on open-source data and open-source software tools. The use of remote sensing data will help the low trophic aquaculture industry to expand their activities to new suitable areas.

2. Materials and Methods

2.1. Study Area

The Ebro delta extends 25 km from the mainland and forms two embayments, Alfacs and Fangar (Figure 2- 1). Fangar Bay, located in the north, has an extension of 12 km^2 and a mean depth of 2 m, it contains 16 × 106 m^3 of seawater, and the mouth of the Bay is 1 km wide. Alfacs Bay is in the south, its extension is 50 km^2, the mean depth is 4 m, it contains 200 × 10^6 m^3 of seawater, and its mouth is 3 km wide. Seawater temperature inside the embayments presents wide fluctuations in comparison to open waters. Both bays receive freshwater inputs from the agriculture drainage channels, favoring stratification during calm conditions (Camp and Delgado, 1987). The average salinity is similar in both bays: 34.6 in Fangar Bay and 34.8 in Alfacs. Freshwater inputs are 228 × 10^6 m^3/year and 365 × 10^6 m^3/year in Fangar and Alfacs, respectively. Water renewal time is approximately 1–2 days in Fangar and 10 days in Alfacs Bay (Camp and Delgado, 1987). Chlorophyll-a concentration increases during wind events due to horizontal mixing and bottom resuspension (Balsells et al., 2021). Both embayments currently hold mussel and oyster production; the location of mussels and oyster rafts is shown in Figure 2- 2.

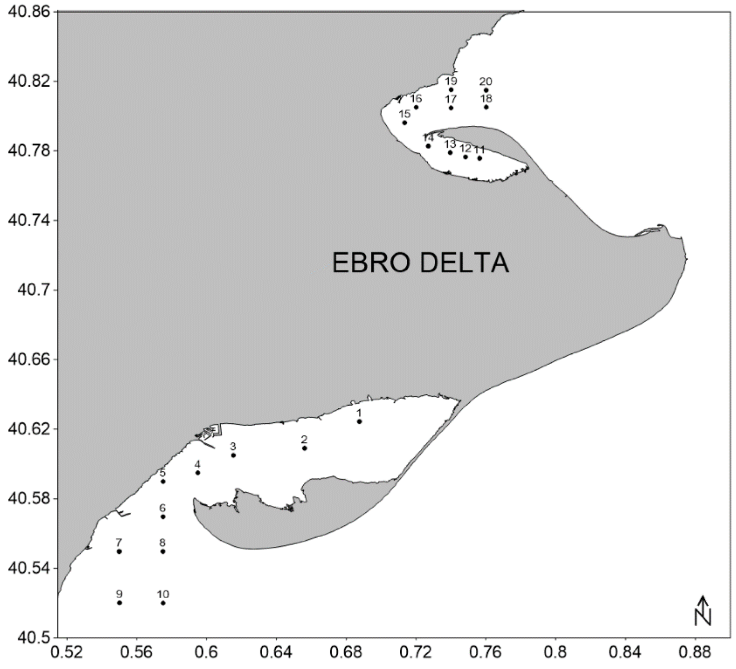


Figure 2- 1. Map showing the location of the sampling stations that were visited for in situ measurements and collection of water samples. Sampling stations in the southern area are numbered from 1 to 10, sampling stations in the northern area are numbered from 11 to 20.

Figure 2- 2. Map showing the extent of the area of study divided in the area inside (AI, FI) and outside the coastal embayments. The external area is divided into different polygons (A1–A6, F1–F6).

2.2. *In Situ* Measurements

Sampling cruises were conducted, coinciding with a Sentinel-2 (S2) pass when the weather forecast was favorable. A total of 17 sampling cruises were carried out during the period 2 September 2020 to 27 October 2021, 9 of them covering the northern area and 8 for the southern area of the Ebro delta (western Mediterranean). Ten sampling stations were visited during each cruise, including waters inside and outside the bays. The location of the 20 sampling stations is shown in Figure 1. At each station, bottom depth, and the Secchi disk depth (ZSD) were measured; the profiles of temperature and salinity were obtained using a SeaBird19plus CTD and water samples were taken for chlorophyll-a analysis. Upon arrival at the laboratory, water samples were filtered through Whatman® glass microfiber filters, Grade GF/F (0.7 μm) 47 mm, using low vacuum. The filters were maintained at -80 °C until analysis. Filter contents were extracted in acetone 90%, the absorbance of the extract was measured in a Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer, and the chlorophyll-a concentration was calculated using the formula Chlorophyll-a = 11.85 E664 − 1.54 E647 − 0.08 E630 (Jeffrey and Humphrey, 1975).

2.3. Maps of Chlorophyll-a from Sentinel-2

The Sentinel-2 (S2) constellation consists of two satellites (S2A and S2B) operated by The European Space Agency (ESA). Each satellite has on-board the MultiSpectral Instrument (S2-MSI). The S2-MSI Level-1 (L1C, Top of atmosphere) imagery includes 13 spectral bands centered at different wavelengths from 443 nm to 2200 nm with different spatial resolutions (10, 20, and 60 m). All available S2-L1C images between 1 October 2019 and 30 September 2021 were downloaded from the Copernicus Services Data Hub (https://cophub.copernicus.eu/dhus/#/home, accessed on 14 July 2022). The only orbit completely covering the study area was orbit #51. Every day the data from this orbit were available, the four tiles needed to cover the area were downloaded. This amounted to a total of 560 images (140 images per tile), covering a surface of 100 × 100 km^2 for each tile.

Each image was visually checked for clouds and shadows over the region of study, leading to the rejection of 350 images (62.5 %), implying the rejection of 46 % of dates with chlorophyll-a in situ data. Due to project administrative deadlines, only images until September 2021 were used to accomplish the study.

The pre-processing of S2 imagery was performed through the Graphic Processing Tool (GTP) of SNAP v8.0 (SNAP-ESA) and Rcore 3.6 (RCoreTeam). For atmospheric correction of S2-MSI L1C imagery, the Case 2 Regional Coast Colour (C2RCC) and the C2X-COMPLEX (C2XC) processors (C2-Nets) (Brockmann et al., 2016) included in SNAP were applied on all valid S2 images. These processors are based on a multi-sensor per-pixel artificial neural network (NN) method, and differ in their training ranges of inherent optical properties (Warren et al., 2019). The parametrization for the atmospheric correction of each image included: (a) pressure (hPa) from NCEP/DOE Reanalysis II data provided by the NOAA PSL, Boulder, Colorado, USA, from their website at https://psl.noaa.gov (accessed on 16 July 2022) (Kanamitsu et al., 2002); (b) atmospheric ozone in Dobson units (DU) from the Aura OMI NASA dataset (NASA Goddard Space Flight Center), downloaded for each location and date from https://oceancolor.gsfc.nasa.gov/ (accessed on 17 July 2022) (NASA Ocean Color Data). Surface seawater temperature and salinity were obtained from the sampling cruises. For land/water segmentation, the valid pixel expression was set as a threshold on the short-wave infrared (SWIR) band of S2-MSI L1C images centered at 1600 nm (B11). The threshold was defined independently for each image with the triangle threshold method. From each C2-Net, remote sensing reflectance (Rrs) of visible and near infrared bands and the pigment absorption product (apig) were generated. C2-Nets flags, which include codes for quality control of pixels, were also exported. For C2RCC and C2XC, independently, images from the same date were merged when more than one tile were available.

A procedure to select match-ups between chlorophyll-a concentration measured from water samples and S2 Rrs was performed for the two C2-Nets. This procedure involves several steps. Firstly a 3 × 3 pixel window, centered at the coordinates of in situ measurements, was extracted for each date and sampling location, and C2-nets were quality-checked in all extracted pixels by applying the recommended flags (Pereira-Sandoval et al., 2019). Then, flagged pixels as well as pixels with negative Rrs at bands B1, B2, B3, and B4 were removed from the analysis, as recommended by Cui et al. (Cui et al., 2010). The number of remaining pixels within each pixel window was checked; windows with less than 5 remaining pixels were removed from the analysis. Outliers were defined through Boxplot analysis applied to each available pixel window and spectral band (B1-B7 and B8A). Finally, the remaining pixel windows with less than 5 valid pixels were removed from the analysis.

A set of band combinations in the form of spectral indices were computed for all valid Rrs match-ups (Table 2- 1). These spectral indices include visible and red edge S2 spectral bands (B1 to B6; ~443 nm to ~740 nm), exploiting specific chlorophyll-a absorption peaks in the blue and red spectral regions. The red edge bands are used for mitigating the effect of absorption by non-algal particles, yellow substances, and backscattering. For each C2-Net, chlorophyll-a was modelled with all the computed spectral indices and the apig band. Seventy percent of the data were used for model calibration (cal) and 30% for model validation (val). The cal/val datasets were generated randomly. Models were developed for raw and log-transformed Rrs/apig and chlorophyll-a data. Linear, linear piecewise (1 breakpoint), polynomial (2nd, 3rd, and 4th order), logarithmic, power, and exponential models were tested. The entire process was iterated 100 times with varying cal/val datasets. For each model (C2-Net × Band Combination × Type of fitting), performance was evaluated by means of the following statistics: mean average error (MAE), root mean squared error (RMSE), average percentage difference (APD), BIAS, and Pearson's r.

Table 2- 1. Spectral bands and spectral combinations tested for chlorophyll-a estimation.

Bands combination	Reference	Equations
Red to Green ratio (RG)	(Cairo et al., 2020)	Rrs(B4)/Rrs(B3)
Blue to Green ratio (BG)	Based on OC2 (O'Reilly and Werdell, 2019)	Rrs (B2)/Rrs(B3)
Blue to Green ratio 2 (BG2)	Based on OC3 (O'Reilly and Werdell, 2019)	max (Rrs (B1), Rrs(B2))/Rrs(B3)
Red Edge 1 to Blue (REB1)	(O'Reilly and Werdell, 2019)	Rrs (B5)/Rrs(B2)
Red Edge 1 to Green (REG1)	(Cairo et al., 2020)	Rrs (B5)/Rrs(B3)
Red Edge 1 to Red (RER)	(Cairo et al., 2020) (Gitelson et al., 2011)	Rrs (B5)/Rrs(B4)
Red Edge 2 to Green (REG2)	(Cairo et al., 2020)	Rrs (B6)/Rrs(B3)
GIT (3 band model)	(Gitelson et al., 2011)	(1/Rrs(B4) - 1/Rrs(B5))*Rrs(B6)
Normalized Difference Chlorophyll Index (NDCI)	(Mishra and Mishra, 2012)	(Rrs(B6) - Rrs(B5))/(Rrs(B6) + Rrs(B5))
apig (*apig*)	(Niroumand-Jadidi et al., 2021)	*apig*

To generate the maps of chlorophyll-a, all flagged pixels (atmospheric correction flagging) were removed, as were pixels with negative Rrs in any of the spectral bands of each valid S2 image processed with the C2-Nets. The best performant model was selected according to lowest MAE, APD, RMSE, and BIAS, in this order, and it was applied to all valid images and pixels. Pixels with negative chlorophyll-a values were set to 0 mg/m3 and pixels with unreliably high chlorophyll-a concentration (>30 mg/m^3) were removed. Pixels corresponding to the mussel farm structures or rafts were masked out using an available shapefile to mitigate/avoid pixel mixing problems.

2.4. Carrying Capacity Model

For the analysis and extraction of data from the generated images, QGIS 3.24 was used (QGIS.org, 2022). The area of interest corresponding to the masked images was divided into 2 different regions inside and outside the two coastal embayments (Figure 2- 2). The studied area comprises 37.5 km^2 inside and 37.8 km^2 outside Alfacs Bay (southern area) and 2.7 km^2 inside and 19.7 km^2 outside Fangar Bay (northern area). The external area was divided into 12 polygons, 6 outside each embayment (Figure 2- 2). The area inside each polygon was 5.5, 7.5, 7, 6.8, 4.8, 6.1 km^2 for A1–A6 and 1.8, 2.5, 2.6, 2.9, 3.8, 6.1 km^2 for F1–F6. Monthly chlorophyll-a concentration was averaged for each area of study, combining the available images for the period. The external areas were divided into different parts (polygons) to evaluate the gradient in chlorophyll-a concentration at different distances from the mouth of the bay. Each polygon was divided into three parts to evaluate the impact of the distance to the shore on the chlorophyll-a concentration.

The chlorophyll-a concentration, retrieved from the S2-L1C images treated with the algorithm calibrated and validated in this study, was used to determine the suitability of the different areas and their carrying capacity. To determine the suitability of each polygon for mussel aquaculture, the DEB model was applied. The model allows us to calculate the amount of energy used for growth and reproduction through the ingestion of the available food using the version from Rosland et al. (2009) and the notation from Kooijman (1986). Mussel ingestion rate is proportional to the surface of the mussel, expressed as structural volume.

$$\dot{P}x = \{\dot{p}_{Xm}\}\, f\ T_d\, V^{2/3} \qquad (1)$$

$$f = \frac{\chi}{\chi + \chi_k} \qquad (2)$$

$$\chi_k = \frac{\{\dot{p}_{Xm}\}\ V^{2/3}}{C_f\, C_R} \qquad (3)$$

$$L = \frac{V^{1/3}}{\delta_m} \qquad (4)$$

$$T_d = \frac{\exp\left(\frac{T_A}{T_I} - \frac{T_A}{T}\right)}{1 + exp\left(\frac{T_{AL}}{T} - \frac{T_{AL}}{T_L}\right) + exp\left(\frac{T_{AH}}{T_H} - \frac{T_{AH}}{T}\right)} \qquad (5)$$

where $\dot{P}x$ is the ingestion rate; the values used for each parameter were obtained from van der Veer et al. (2006) and are shown in Table 2- 2. Clearance rates (C_R) were obtained from Galimany et al. (2011). The measured in situ seawater temperature (°C) at 1 m depth was converted to Kelvin degrees (K). In Equation (2), f is the Michaelis–Menten Equation to scale the ingestion rate to the food concentrations (X); this term scales the amount of food ingested as a function of the food (chlorophyll-a) available. In Equation (4), L is mussel length (cm), where δM is the dimensionless shape coefficient. Td in Equation (5) is the Arrhenius temperature function. The model (Equations (1)–(5)) was computed in Python.

Table 2- 2. Values used for the DEB model.

Parameter	Unit	Definition	Value
$\{\dot{p}_{Xm}\}$	J cm^{-2} d^{-1}	Maximum Surface area-specific assimilation rate	273
χ_k	mg C m^{-3}	Half saturation coefficient	calculated [1]
T_A	K	Arrhenius temperature	5800
T_I	K	Reference temperature	289
T_L	K	Lower boundary of tolerance range	275
T_H	K	Upper boundary of tolerance range	296
T_{AL}	K	Arrhenius temperature for rate of decrease at lower boundary	45430
T_{AH}	K	Arrhenius temperature for rate of decrease at upper boundary	31376
δ_m		Shape coefficient	0.29
V	cm^3	Bivalve volume	0.06

C_f	Jµg^{-1} chla	0.419

^{1} Calculated using Formula (3)

In a previous study (Ibáñez-Solé, 2014), the DEB model was found to better reflect field observations of mussel ingestion in Alfacs Bay, in comparison with the Scope for Growth (SFG).

Carrying capacity was calculated by applying the recommendations of the Aquaculture Stewardship Council (2019). This standard provides indicators for a first approach to characterize the potential of an area. In the present study, the carrying capacity was defined as the number of rafts/ropes/mussels that can be grown in each area and was calculated as a function of the food availability measured through the chlorophyll-a concentration and the renewal time of the water masses. Chlorophyll-a concentration was obtained from the S2 images treated with the algorithm in this study, and the renewal time was calculated using the current speed at the different external areas.

$$CC(mussels) = \frac{\left[\frac{Chla(mg) * 0.75}{Cr\left(\frac{l}{h}, date\right)_{mussels} * Chla\left(\frac{mg}{l}, date\right)}\right].}{Tr(h)} \quad (6)$$

$$CC(ropes) = \frac{CC(mussels)}{rope} \quad (7)$$

In formula (6), $Tr(h)$ is the renewal time of the water in each area, $Chla(mg)$ is the total amount of chlorophyll-a in the whole volume of each area, and $Chla\left(\frac{mg}{l}\right)$ is the chlorophyll-a concentration of each area obtained from the Satellite images.

$$Chla(mg) = Volume\ (l)\ x\ Chla\left(\frac{mg}{l}\right) \ (8)$$

Different values of clearance rate, rate $Cr\left(\frac{l}{h}, date\right)_{mussels}$ were applied by selecting the corresponding values for each time of the year. The percentage of chlorophyll-a available for the cultivated mussels was assumed to be 0.75, and 0.25 was the remnant percentage. A standardized length of 3 m per rope that holds 1250 mussels was also used. These are the usual characteristics of the mussel ropes used in the Ebro delta. For the external areas, the average renewal times were calculated from the Copernicus Marine Service (Clementi et al., 2021) using data from the years 2019 to 2021. For all areas, the volume was calculated for 3 m of depth as standard length of mussel ropes. The renewal times used for the areas inside the embayments were obtained from the bibliography (Delgado, 1987). We have not included primary production time because renewal times are short in the external areas.

The following diagram Figure 2- 3 shows the relation between in situ data, remote sensing data, and the different models used in this study.

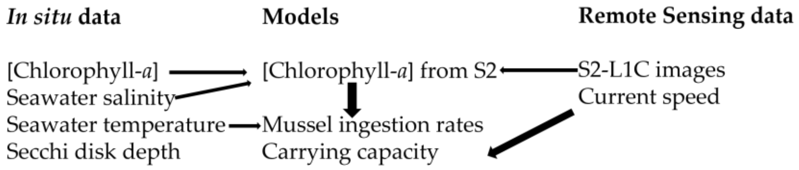


Figure 2- 3. Diagram showing the relation between in situ data, remote sensing data, and the different models.

3. Results

3.1. *In Situ* Measurements

The results from the *in situ* measurements in October 2021 were not used for the calibration and validation of the satellite images but are included here to provide a wider view of the in situ environmental conditions of the area. The average depth of the 16 sampling stations was 7.7 ± 0.2 m (Mean ± Std. Error) and the range was 3.7–14.8 m. The average Secchi depth was 3.5 ± 0.2 m (Mean ± Std. Error) and the range was 1.3–10.4 m (Figure 2S- 1). The average chlorophyll-a concentration in the water samples was 2.4 ± 0.1 mg/m^3 (Mean ± Std. Error) and the range was 0.1-8.9 mg/m^3 (Figure 2- 4). There was a statistically significant difference in chlorophyll-a concentration and Secchi disk depth ($p \leq 0.001$) between the interior and the exterior sampling stations from the whole studied area (Kruskal–Wallis One Way Analysis of Variance on Ranks). In the southern area, there was no statistically significant difference in the mean values of chlorophyll-a concentration among the different sampling stations ($p = 0.118$) and dates ($p = 0.251$) (Two Way Analysis of Variance). In the northern area, there was a statistically significant difference ($p \leq 0.001$) between sampling stations and dates.

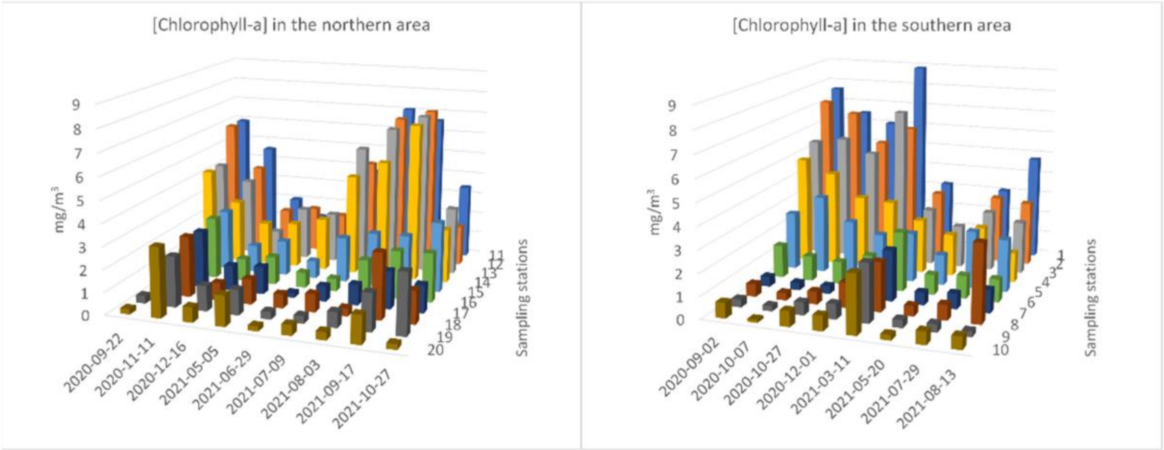


Figure 2- 4. Concentration of chlorophyll-a (mg/m3) at the different sampling stations.

The range in seawater temperature was 10.9–28.2 °C in the northern area outside Fangar Bay and 11.7–29.2 °C in the southern area outside Alfacs Bay. Minimum values were registered in January 2020 and maximum values in August 2020 (Figure 2- 5). Higher temperature is measured in the southern area in comparison to the northern area.

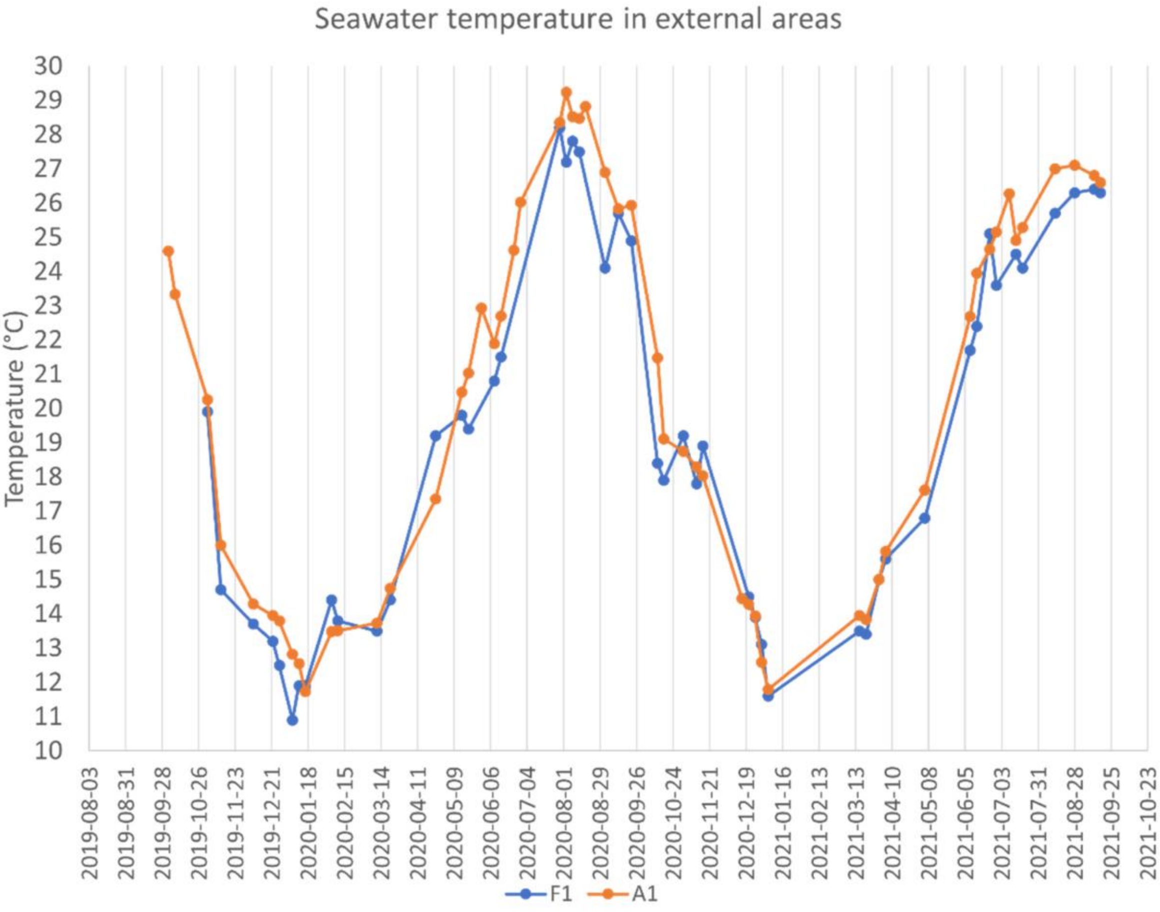


Figure 2- 5. Seawater temperature (°C) in the external areas of the Ebro delta during the period of study.

3.2. Maps of Chlorophyll-a from Sentinel-2

Models performed better with C2XC than with C2RCC, particularly coupled with band combinations including spectral bands in the blue or green, and red or red edge regions. The best performance models for each C2-Net and band combinations are shown in Table 3. The best model for chlorophyll-a estimation was found with the C2XC processor, applying the REB1 band ratio (see Table 2- 3) with a 2nd polynomial fitting ([chlorophyll-a] = −0.615 + 10.88 × REB1 + 8.704 × REB12).

Table 2- 3. Best performing models per C2-Net and spectral band combination.

Bands	C2-Nets	FIT [1]	MAE	APD	RMSE	BIAS	r
RG	C2RCC	Pm4	0.736	39.843	1.107	-0.075	0.820
RG	C2XC	Pm4	0.703	59.572	0.949	0.083	0.870
BG	C2RCC	Lm	0.726	39.815	1.107	-0.070	0.817
BG	C2XC	LLPm3	0.734	39.634	1.120	-0.102	0.816
BG2	C2RCC	Lm	0.751	59.752	1.013	-0.061	0.850
BG2	C2XC	LLPm3	0.734	39.634	1.120	-0.102	0.816
REB1	C2RCC	Lm	0.741	39.530	1.101	0.018	0.820
REB1	C2XC	Pm2	0.598	36.216	0.887	0.054	0.887
REG1	C2RCC	Lm	0.738	38.898	1.097	-0.027	0.820
REG1	C2XC	Pm4	0.616	42.844	0.878	-0.022	0.889
RER	C2RCC	Lm	0.745	51.303	1.090	-0.056	0.825
RER	C2XC	LLPm4	1.005	73.194	1.428	-0.263	0.682
REG2	C2RCC	Lm	0.742	51.294	1.047	0.039	0.839
REG2	C2XC	LLPm2	0.669	50.039	0.947	-0.048	0.870
GIT	C2RCC	Lm	0.748	54.933	1.038	0.019	0.842
GIT	C2XC	LLPm4	1.052	79.078	1.486	-0.223	0.644
NDCI	C2RCC	Lm	0.749	42.445	1.104	0.095	0.820
NDCI	C2XC	Pm4	1.358	113.510	1.880	-0.297	0.251
apig	C2RCC	Lm	0.753	59.581	1.010	-0.017	0.850
apig	C2XC	LLPm4	1.064	78.601	1.483	-0.193	0.661

[1] Lm: Linear model; PmX: X degree polynomial; LLPmX: Log-Log X degree polynomial.

The calibration and validation plots, as well as the fit between observed and estimated chlorophyll-a concentration values, are presented in Figure 2- 6.

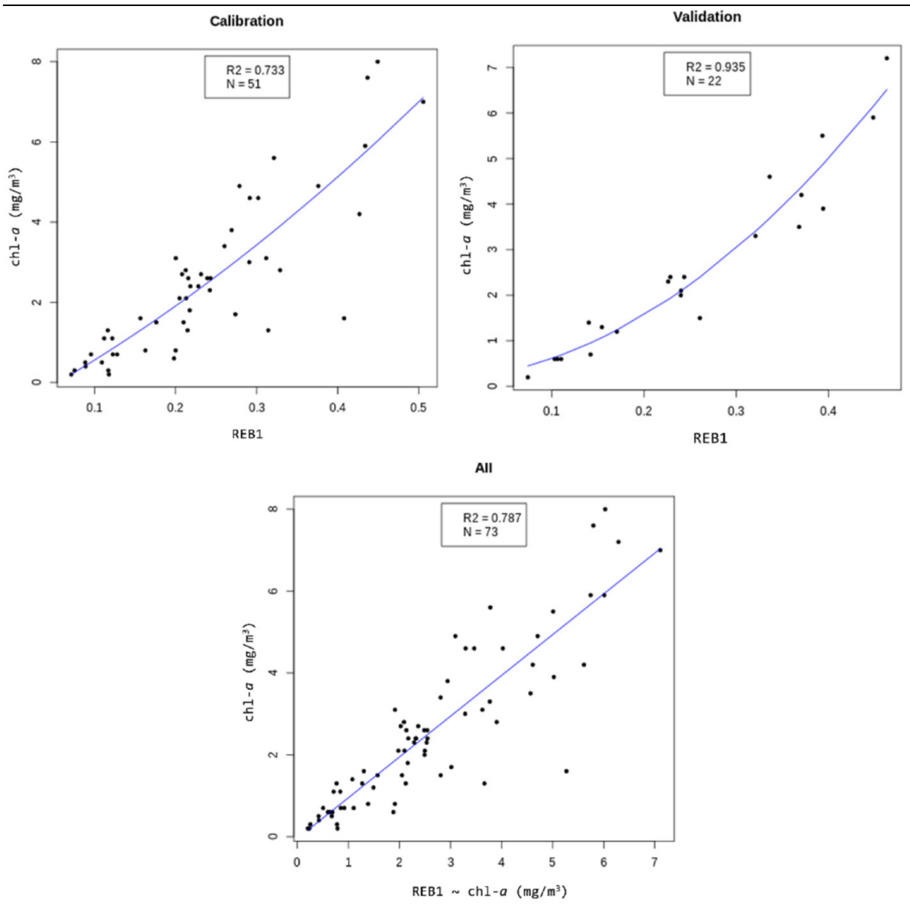


Figure 2- 6. Calibration, validation, and linear regression of the best model.

Maps of chlorophyll-a concentration from 56 different dates (Figure 2- 7) along the period of study were generated using the methodology described in Section 2.3. A good agreement between estimated and observed chlorophyll-a concentration was observed within the sampling dates and the dynamic range of in situ data. However, unreliable high chlorophyll-a concentration was retrieved in some scenarios, such as the image of the 5 February 2020 (Figure 2- 7), captured ~2 weeks after an extreme coastal storm. In those scenarios, dark brownish waters were visible in the RGB image composite, suggesting greater total suspended matter concentration and turbidity, leading to more optically complex waters.

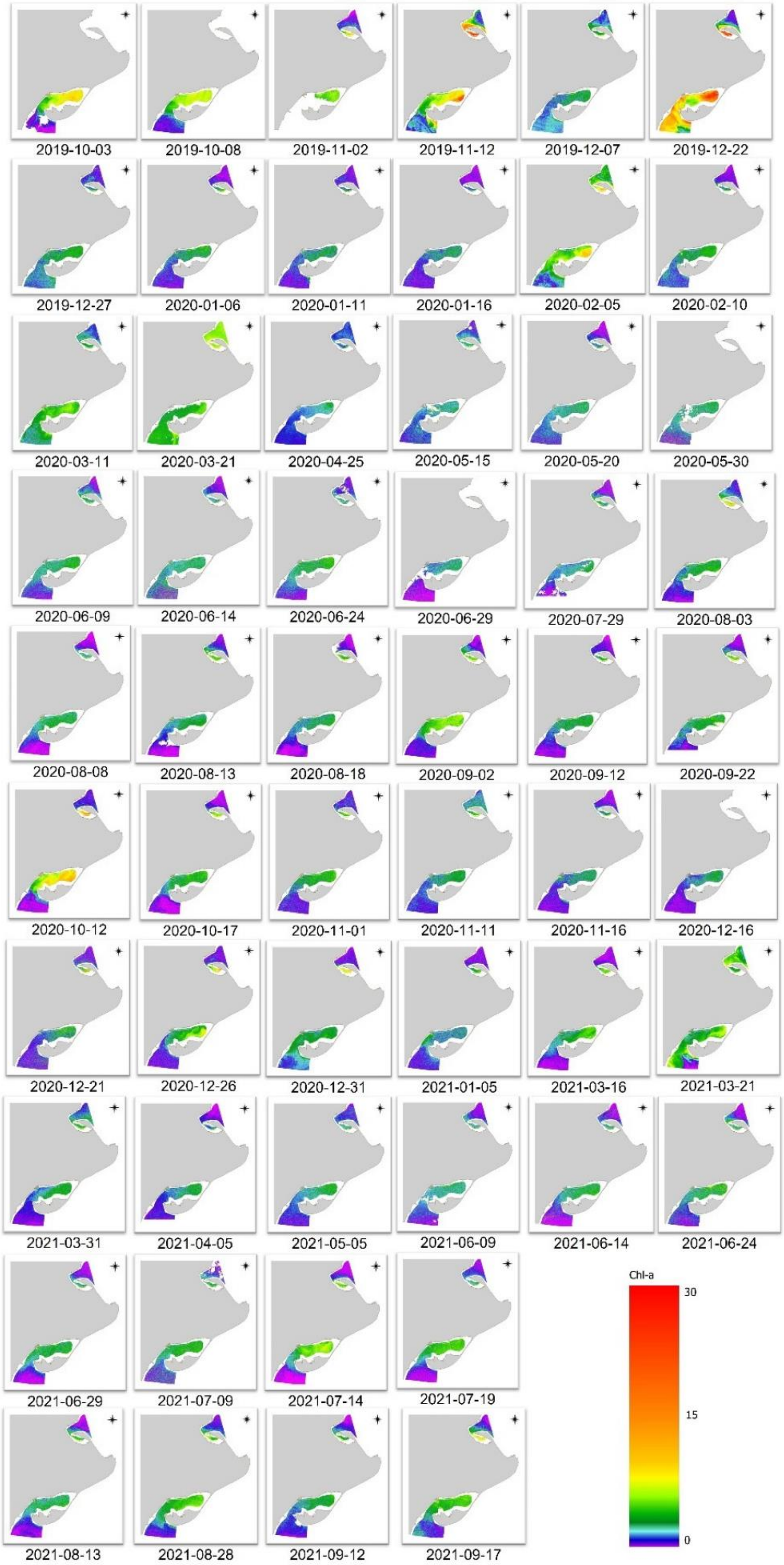


Figure 2- 7. Maps of chlorophyll-a concentration from the area of study (October 2019 to September 2021) derived from Sentinel-2.

3.3. Carrying Capacity Model

Monthly averages of chlorophyll-a concentration for the areas AI, A1–A6 together and FI, F1–F6 together are shown in Figure 2- 8. Higher values of chlorophyll-a concentration were observed inside the two coastal embayments in comparison to the external area. The highest values occurred in Fangar Bay during autumn (13.6 mg/m^3) and summer (8.5 mg/m^3).

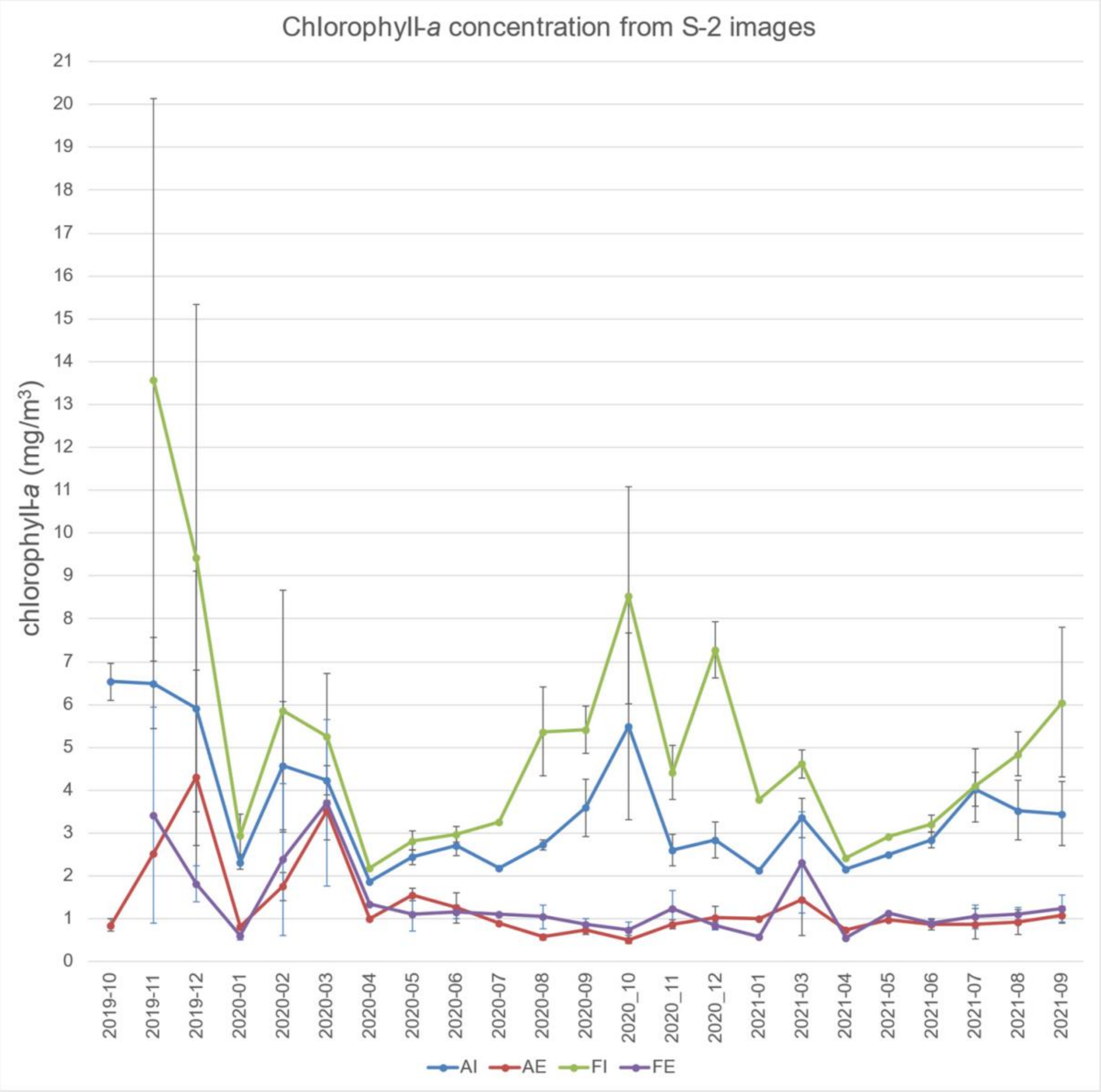
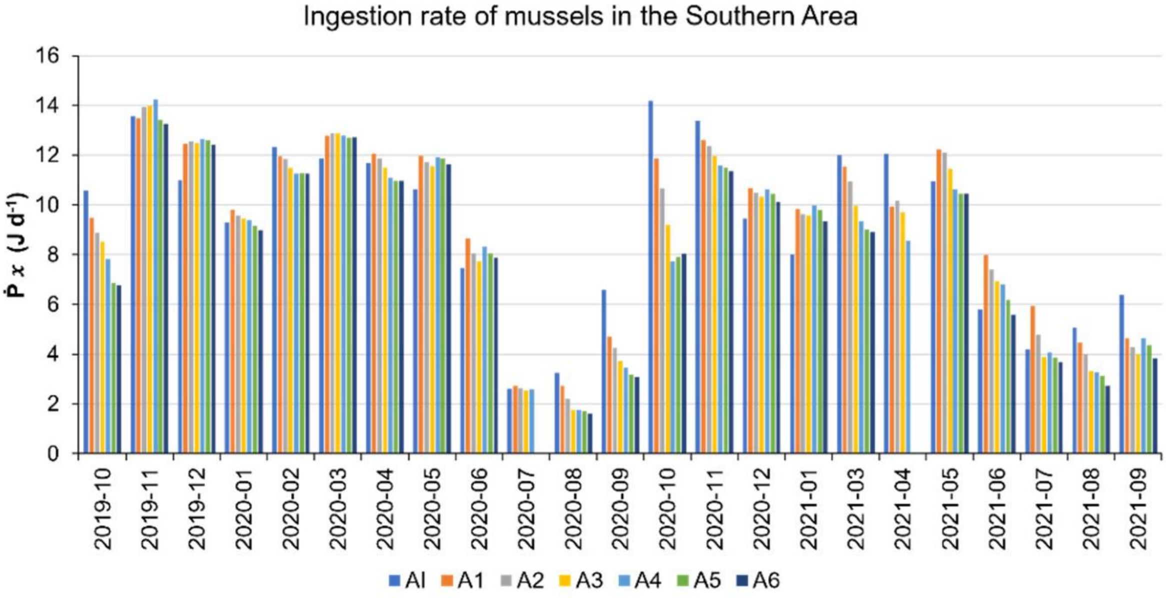


Figure 2- 8. Monthly chlorophyll-a concentration (Average ± Std. Error), from Sentinel-2 images, inside and outside the coastal embayments (AI: Alfacs interior, AE: Alfacs exterior, FI: Fangar Interior, FE: Fangar exterior).

In the north (F1–F6), the maximum averaged chlorophyll-a value per area and date (13.8 mg/m^3) was detected in November 2019 in F1; in the south (A1–A6) it was detected in A2 in December 2019 (12.1 mg/m^3). There are statistical differences between the different areas (Chi-square = 122, DF = 3, $p < 0.001$). The differences are between FI vs. AE, FI vs. FE, FI vs. AI, AI vs. AE, and AI vs. FE (Tukey test $p < 0.05$), but not between FE vs. AE (Tukey test $p > 0.05$). The averaged concentration of chlorophyll-a per date and polygon outside the embayments is shown in Figure S2. The minimum of the averaged values per image, external area, and date (0.12 mg/m^3) was measured in July 2021 in A6 (southern area). In the same image, clouds where present in some of the areas, while in other areas of the same image the chlorophyll-a concentration could be measured. There are statistical differences between the different polygons (Tukey test $p < 0.05$); the results of the statistical analysis are shown in Figure 2S- 3. The gradient in chlorophyll-a concentration in the external areas at different distances from the bay's mouth can be observed in Figure 2S- 4.

In the northern area outside Fangar Bay, apart from the gradient observed in chlorophyll-a at different distances from the mouth, a second gradient is observed inside each of the external areas at different distances from the shore, the closest areas to the shore being the richest in chlorophyll-a (Figure 2S-3). A gradient is also observed in the southern areas, but in the opposite way. In polygons A1–A4, the areas closest to the shore contain less chlorophyll-a than those farther from the shore, except in polygons A5–A6, where the same pattern observed in the northern area occurs.

Ingestion rates ($\dot{P}_x$) from October 2019 to September 2021 for the different polygons of the southern area and from November 2019 to September 2021 for the northern area are shown in Figure 2- 9. In the external southern area, the mean value of P_x was 8.7 ± 0.3 Jd^{-1} and the range was 1.6–14.2 Jd^{-1}. The highest P_x occurred from October 2019 to June 2020, when the range was 6.8–14.2 Jd^{-1}; it decreased to values ≤ 8 Jd^{-1} from July to September 2020, increasing in October 2020 to values > 8 Jd^{-1} until May 2021, and decreasing again in June to September 2021 to values ≤ 8 Jd^{-1}. In the external northern area, the mean value of P_x was 7.7 ± 3.1 Jd^{-1} and the range was 1.7–12.5 Jd^{-1}. In this area, the range was 6–12.5 Jd^{-1} from November 2019 to June 2020, decreasing to values < 6 Jd^{-1} from July to September 2020, increasing in October 2020 to values > 6 Jd^{-1} until May to June 2021, decreasing another time in July to September 2021. In both the northern and the southern area, there was a statistically significant difference ($p \leq 0.001$; Two Way Analysis of Variance, General Linear Model) among the values for different dates and polygons.



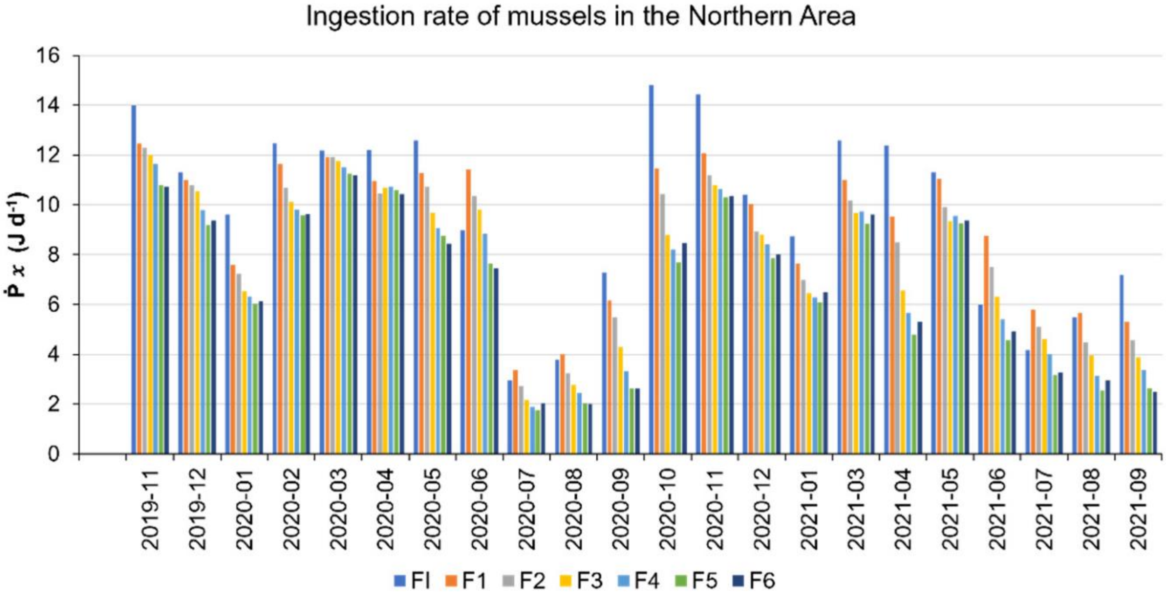


Figure 2- 9. Monthly average of the ingestion rates of mussels, $\dot{P}_x$ (J d^{-1}), inside the embayments and for the different polygons outside.

The carrying capacity (CC) calculated for each area is shown in Table 4 as the number of mussel rafts that can be held; a standardized raft in this study contains 1100 ropes of mussels that are 3 m in length. The renewal time for each area and the clearance rate time (h) calculated for the number of mussels and the chlorophyll-a stock of the area are also shown in Table 4. Renewal times are shorter than the clearance rate calculated because 0.75 of the chlorophyll-a stock was selected as available for CC. The renewal time used in Table 2- 4 is the averaged renewal time for the year in each area. Because the renewal time is variable over the year, the CC was also modeled for different renewal times (Figure 2- 10). We can observe that CC tends to stabilize at higher renewal times, depending on the internal areas below 50 rafts and the external areas below 200 rafts. The shaded red area in Figure 2- 10 corresponds to the renewal time range described in previous studies and the red line is the CC calculated for this renewal time. In the external areas we observed the same exponential decreasing result.

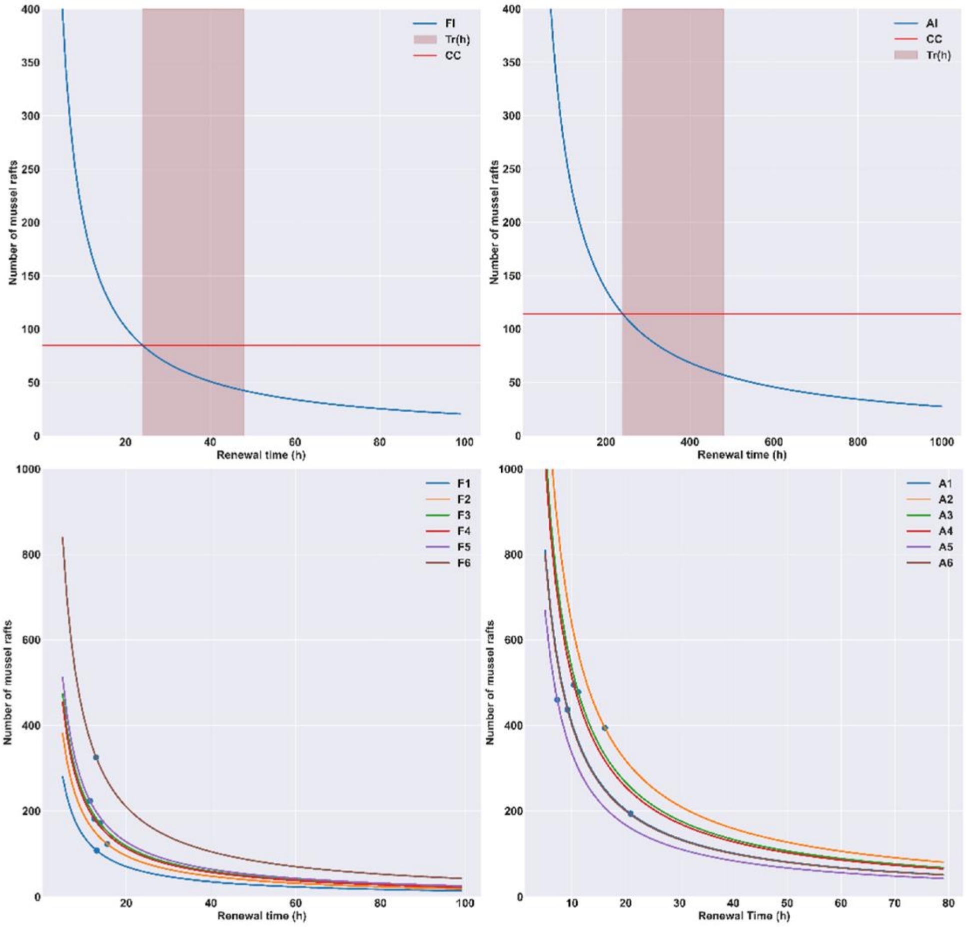


Figure 2- 10. Modeled number of rafts that each area can hold depending on the renewal time (h). The interior areas (FI, AI) are shown at the top of the image and the external areas (F1–F6, A1–A6) at the bottom. The horizontal red line represents the carrying capacity (CC) calculated for the average renewal time in hours (Tr) of the areas (FI, AI). The shaded area in FI, AI corresponds to the average renewal time of each embayment. The dots on the lines at the bottom of the figure correspond to the CC calculated for the average renewal time of each external area (F1–F6, A1–A6).

Table 2- 4. Carrying capacity (CC) calculated for each area together with the corresponding surface and renewal times (Tr) and Clearance rate times (Cr). Note that the surface in AI and FI corresponds to the masked areas not to the whole surface of the embayments.

Area code	Surface (m²)	Tr (h)	CC (number of mussel rafts)	Cr (h)
AI	3.7×10^{7}	240.5	114	331
A1	5.5×10^{6}	20.9	194	29
A2	7.5×10^{6}	16.1	352	19

A3	7 x 10^{6}	11.1	478	15
A4	6.8 x 10^{6}	10.3	494	14
A5	4.8 x 10^{6}	7.3	460	10
A6	6.1 x 10^{6}	9.2	437	14
FI	2.7 x 10^{6}	24.5	85	64
F1	1.8 x 10^{6}	13	107	17
F2	2.5 x 10^{6}	15.5	120	21
F3	2.6 x 10^{6}	13.8	107	15
F4	2.9 x 10^{6}	12.5	174	17
F5	3.8 x 10^{6}	11.4	223	17
F6	6.1 x 10^{6}	12.9	325	19

4. Discussion

The values of *in situ* Secchi disk depth and chlorophyll-a concentration measured in this study (2019–2021) differed slightly from those found in a previous study (Delgado, 1987) conducted during the period 1982–1983. The Secchi disk depth (ZSD), at that time, was measured at some of the same sampling stations used for our study. The values obtained in (Delgado, 1987) were 0.7–1.9 m deeper than our measurements when comparing the average values for each station. The same authors measured chlorophyll-a concentration and found yearly averages for the inner areas of 3.2 mg/m^3 for AI and 3.44 mg/m^3 for FI; these values are slightly lower than those found in our study: 5.2 mg/m^3 for AI and 4 mg/m^3 for FI. Therefore, the differences in Secchi disk depth can be partially explained by differences in chlorophyll-a concentration. Secchi disk depth (ZSD) is influenced by the phytoplankton biomass and other optical variables such as the colored dissolved organic matter (CDOM) and suspended particulate matter (SPM). Resuspension of sediments and the brownification of coastal waters influence ZSD, and therefore its relationship with chlorophyll-a concentration is not a straight inverse relation. The same authors detected maximum chlorophyll-a values in June–July (3.5–11 mg/m^3) and September–October (25 mg/m^3), and values in the range of 1–3.7 mg/m^3 for the rest of the year. The maximum chlorophyll-a concentration in water samples measured in our study was 8.9 mg/m^3 in December 2020 (AI). Higher values were observed from the satellite images. It is important to highlight that the values of chlorophyll-a retrieved from the Sentinel-2 images for the different polygons located at different distances from the mouth of the bays reflect the gradients observed in the *in situ* measurements of chlorophyll-a concentration.

Different methods exist for atmospheric correction in coastal waters, and some of them (C2-Nets, iCOR) are available in open-source tools such as SNAP. This feature opens the door to non-specialized users to generate atmospherically corrected S2 images by using this tool and following the proposed methodology. The model proposed here for mapping chlorophyll-a concentration, and the pixels quality control procedure, can also be implemented using the band-math's functions provided by this tool.

The proposed methodology allows one to estimate chlorophyll-a concentration from Sentinel-2 with an accuracy higher than 70 % in most cases (Table 2- 3 and Figure 2- 6). The best performant model was achieved with C2XC, which showed more consistent and accurate Rrs estimates than C2RCC in prior research (Soriano-Gonzalez et al., 2022). However, empirical algorithms, such as the one used, can be expected to perform well only inside their range and for the area they were derived for. They are also limited in their ability to discriminate between non-unique signals from parameters that may be covariant, for example TSM and chlorophyll-a concentration (Matthews, 2011). The developed model is less reliable in conditions of high concentration of TSM and/or CDOM, which may occur after strong winds or storms (increased water turbulence, sediment resuspension, land runoff), as observed on 5 February 2020 (Figure 2- 7). In these cases, the selected model tends to overestimate chlorophyll-a concentration because the red edge reflectance increases at high TSM values overlapping the absorption of chlorophyll-a in the blue region of the spectrum, which is also affected by greater CDOM concentration (CDOM strongly absorbs light up to 500 nm) (Ligi et al., 2017; Niroumand-Jadidi et al., 2021). In these cases, other empirical or semi-analytical algorithms may be more accurate. Further research should focus on the development of a multi-algorithm blending approach, which has proven to be more suitable across different types of water optical properties (Moore et al., 2014), albeit it will involve a more complex procedure. The development of an algorithm switching-based method requires the accurate definition of the optical water type pixel by pixel, accounting for the spectral shape, magnitude, and distinctive Rrs spectral features (Spyrakos et al., 2018; Uudeberg et al., 2019) and considering the uncertainty of the Rrs retrieval from C2-Nets across different optical water types (Soriano-Gonzalez et al., 2022). In addition, in situ data covering all different scenarios are desirable, which may be difficult to reach due to complex logistics and meteorological limitations (i.e., cloud coverage), as occurred in our study.

The gradient observed in chlorophyll-a concentration from the inner areas towards the open sea is also observed in the values of ingestion rate ($\dot{P}_x$). The values of $\dot{P}_x$. integrate the effect of chlorophyll-a concentration and seawater temperature on mussel physiology. Lower seawater temperature in summer in open waters in comparison to the inner areas could compensate the effect of having less phytoplankton available in these waters, obtaining similar or even better growth when high seawater temperature decreases mussel filtration rates. When we observe the mean values for the period of study in each area, in the north, we can see that the gradient decreases from 9.1 Jd^{-1} to 6.7 Jd^{-1} from area F1 to area F5, and it increases slightly to 6.9 Jd^{-1} in area F6. The gradient is less stepped in the southern area, from 9.3 Jd^{-1} in A1 to 8.3 Jd^{-1} in A6. We can employ the value of $\dot{P}_x$. to rank the suitability of the different polygons for mussel aquaculture; the most suitable would be those with the highest $\dot{P}_x$, which are A1 and F1 followed by A2–5. The less suitable would be F3–6. We have based our model on the chlorophyll-a concentration measured in each area. The DEB model reflects well the decrease in food ingestion during summer months due to limiting seawater temperatures

(Anestis et al., 2007). The use of chlorophyll-a as a proxy does not consider changes in food quality due, for example, to the presence of certain phytoplankton species with a different nutritional value. Some authors have detected gaps in DEB model parametrization and proposed to improve seston characterization (Filgueira et al., 2020; Filgueira et al., 2019), which should be tackled in further research.

A shellfish farm may exceed the ecological carrying capacity when the removal of phytoplankton biomass exceeds the renewal, resulting in a phytoplankton depleted water mass. In the proposed CC model, the renewal time of each area is shorter than the clearance rate time, thus complying with the ASC bivalve standard (Aquaculture Stewardship Council, 2019) that recommends comparing how long it takes to clear the body (CT) of water with the renewal time (RT), the ratio CT/RT should be >1. The same standard recommends that in locations where CT < RT, then the ratio CT/PPT should be >3. PPT is the number of days required for the replacement of phytoplankton biomass (PB) in the water body considering phytoplankton growth (PB/PPP; PPP = phytoplankton primary production). When the clearance rate is higher than the renewal rate plus the primary production, the body of water is depleted of phytoplankton and the bivalve stock will have less food available. Other authors suggest employing the regulation ratio defined as a fraction of the phytoplankton turnover rate RR = (1/CT)/(1/PT), where PT is the time it takes to renew the phytoplankton stock in an area (Filgueira et al., 2019; Smaal and van Duren, 2019). We have applied the same formula for CC in the internal and external areas without considering primary production time. Renewal time in the internal areas is longer and it would be appropriate to include primary production time in the calculations. There are already mussel and oyster farms inside the embayments, therefore the chlorophyll-a concentration measured is affected already by the consumption of the bivalves from these farms. More complex models are needed to assess, with more detail, the carrying capacity inside the embayments. Renewal times are not uniform inside the embayments where hydrodynamic models such as the Regional Ocean Model System (ROMS) coupled to a biogeochemical nutrient-phytoplankton-zooplankton-detritus model (NPZD) such as in Dabrowski et al. (2013) will provide a more accurate evaluation. Hydrodynamical modeling coupled to ecological modeling has been applied in other geographical areas to decide the most appropriate locations of the bivalve farms inside an embayment to avoid negative consequences for the ecosystem (Filgueira et al., 2021). We have shown how small changes in renewal time affect the carrying capacity of the different water bodies in our study. The standard from the Aquaculture Stewardship Council (Aquaculture Stewardship Council, 2019) recommends to use retention time calculated as the number of days for tides to flush a volume of water equal to the volume of the area. In the Mediterranean Sea, tides have low amplitude, and so flushing time has to be calculated by other means.

The results on the modeled number of rafts in each area show that these external areas can hold a significant production of mussels in waters that are less impacted by warm events in summer. Mussel growth in these areas may be slower in comparison to the embayments due to lower chlorophyll-a concentration. The differences in seawater temperature measured during this study in summer were 1–2 °C for the same day and depth between internal and external areas; this small difference may be crucial to reduce mussel mortality in summer.

5. Conclusions

The proposed methodology can be reproduced using open-source tools such as SNAP software, enabling end-users to obtain their own maps of chlorophyll-a concentration from Sentinel-2 images. This methodology allows one to estimate chlorophyll-a concentration from Sentinel-2 with an accuracy higher than 70 % in most cases. The best performant model was achieved with C2XC for atmospheric correction of S2-MSI L1C imagery. We have shown that the application of the DEB theory using multispectral remote sensing imagery allows one to rank the suitability of the different areas for shellfish aquaculture. It also allows one to determine the periods of the year with favorable and unfavorable conditions for shellfish growth. The results presented in this study show that the carrying capacity of the different areas is highly variable depending on the renewal times of the water, which at the same time are highly variable during the year. Nevertheless, the external areas located close to the Ebro delta embayments can hold a significant mussel production that would allow the reduction of mussel mortality during summer months due to the lower seawater temperature in these areas. This study shows the potential of the combined use of satellite remote sensing, in situ sampling, and carrying capacity models as a tool for helping the low trophic aquaculture industry to expand their activities to new suitable areas.

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Chapter 3. Trends in toxic phytoplankton in the Mediterranean.

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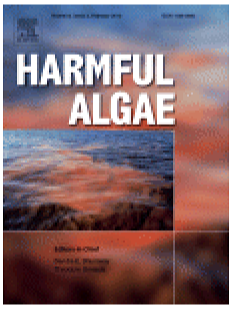

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Highlights

- The Mediterranean Sea harbors more than 80 toxin-producing species, with a few cases of DSP and PSP and about 300 toxic events in 31 years, with an impact mainly on aquaculture.
- The number of toxic species detected in the area has remarkably increased since the 1980s, but with no clear trend in toxin-related harmful events.
- Large amounts of palytoxin-like toxins produced by *Ostreopsis* accumulate along rocky shores in summer since the 1990s, with sporadic problems caused by direct contact or aerosol.
- Non-toxic events such as seawater discolorations and mucilages show wide fluctuations over the years with no clear trends, but represent the main risk in the MS for their possible impact on tourism and recreational activities.

Abstract

We review the spatial distribution of toxic marine microalgal species and the impacts of all types of harmful algal events (Harmful Algal Blooms, HABs) in the Mediterranean Sea (MS), including the Black Sea, the Sea of Marmara, coastal lagoons and transitional waters, based on two databases compiled in the Ocean Biogeographic Information System (OBIS). Eighty-four potentially toxic species have been detected in the MS (2,350 records), of which 16 described from these waters between 1860 and 2014 and a few suspected to have been introduced. More than half of these species (46) produce toxins that may affect human health, the remainders ichthyotoxic substances (29) or other types of toxins (9). Nevertheless, toxicity-related events are not frequent in the MS (308 records in 31 years), and mainly consist of impacts on aquaculture, caused by the dinoflagellates *Dinophysis* and *Alexandrium*, along with a few actual shellfish poisoning cases. *Pseudo-nitzschia* blooms are widespread, but domoic acid in shellfish rarely exceeds regulatory levels. Fish kills are probably less sporadic than reported, representing a problem at a few places along the southern MS coasts and in the Ebro River Delta. Since the last decade of the 20th century, blooms of the benthic dinoflagellates *Ostreopsis* cf. *ovata* have regularly occurred all along rocky shores of the MS, at times with human health problems caused by toxic aerosol. New records of *Gambierdiscus* and *Fukuyoa*, until now reported for the westernmost and easternmost MS coasts, raise concerns about the risk of ciguatera, a syndrome so far known only for subtropical and tropical areas. Recent discoveries are the dinoflagellates *Vulcanodinium rugosum*, responsible for the presence of pinnatoxins in French lagoons' shellfish, and the azaspiracid-producers *Azadinium* spp. Mucilages and discolorations have a major impact on tourism in summer. Reports of toxic species and HABs have apparently increased in the MS over the last half century, which is likely related to the increased awareness and monitoring operations rather than to an actual increase of these phenomena. Indeed, while the case of *Ostreopsis* appears as a sudden upsurge rather than a trend, no actual increase of toxic or noxious events has so far emerged in intensively studied areas, such as the French and Spanish coasts or the Adriatic Sea. Moreover, some cases of decrease are reported, e.g., for *Alexandrium minutum* blooms disappearing from the Harbour of Alexandria. Overall, main HAB risks derive from cases of massive development of microalgal biomass and consequent impacts of reduced coastal water quality on tourism, which represents the largest part of the marine economy along the MS coasts.

Keywords

HABs, Mediterranean Sea, Microalgae, Toxicity, OBIS

1. Introduction

The Mediterranean Sea (MS, from the Latin mare Mediterraneum = the sea surrounded by land) is an enclosed basin surrounded on the north by southern Europe and Anatolia, on the south by North Africa and on the east by the Levant. It occupies an area of approximately 2510,000 km^2 lying between latitudes 30° and 46° N. The narrow and shallow Strait of Gibraltar to the west connects it with the Atlantic Ocean, the Dardanelles to the east with the Black Sea through the Sea of Marmara and the Bosporus, while to the south-east the Suez Canal, opened in 1869 and recently expanded, allows the exchange with the Red Sea. In spite of its geographic position within the northern temperate latitudes, the quite shallow sill (170 m) at the Atlantic boundary blocks the entrance of deep, cold oceanic waters and determines temperate-subtropical conditions in the whole area, with minimum temperatures rarely and only at certain locations going below 12 °C.

The size, location, and morphology of the MS are at the base of its complex physical dynamics with a distinctive thermohaline circulation and permanent or semi-permanent sub-basin gyres. A marked oligotrophy, increasing along both the west-east and the north-south directions, characterizes the MS (Siokou-Frangou et al., 2010). However, along the Mediterranean coasts there are densely populated areas while a number of large rivers with extended catchment basins flow in the MS (e.g., the Po in the northern Adriatic, the Nile in Egypt, the Ebro in Spain, and the Rhone in France). This implies that meso- and eutrophic conditions, and at times pollution, can affect various coastal areas (UNEP-MAP, 2012).

The MS has been the crossroad of various cultures since the very beginning of the human colonization and the development of ancient civilizations. Trading routes, migrations, invasions and the struggle for power have shaped the dynamic history of populations around the basin for millennia. The population grew from 281 million in 1970 to 419 million in 2000 and 472 million in 2010, and is predicted to reach 572 million by 2030. Coastal administrative entities make less than 12 % of the surface area of the Mediterranean countries, but host more than a third of the population of the whole region. Coastal population grew from about 100 million in 1980 to 150 million in 2005 and could reach 200 million by 2030 (UNEP-MAP, 2017).

The MS also represents a unique geographic landscape that generates wealth but requires cooperation among the different countries to preserve the environment and the biological resources. The conservative value of the economic assets of the MS has been estimated to be in the order of US$ 5.6 trillion, generating an annual economic value of US$ 450 billion (Randone et al., 2017). A large fraction of the economic value is represented by tourism and related activities; fisheries come as second but >80% of the fish stock is presently threatened. Aquaculture in the MS has considerably expanded over the last decades reaching about 1.3 million tons in 2009 with an estimated value of US$ 3700 million (Rosa et al., 2012). Most of the marine aquaculture production comes from the north Mediterranean countries, which are also the most intensively monitored, but it is rapidly expanding also in Turkey and Egypt. In spite of the dramatic alteration of habitats, depletion of natural resources and increased number of alien species, the MS is still characterized by high biodiversity in most animal and algal groups and a considerable number of endemic species (Coll et al., 2010).

The rate at which climatic conditions (e.g., surface temperature, heat waves and sea level) have changed in the MS over the last decades is higher than the global average (Cramer et al., 2018). These changes, coupled with increased population size, urbanization and changes in land use at many coastal places, may pose at serious risk the quality of the environment, the quality and quantity of food and consequently the health and safety of the local populations (Cramer et al., 2018). Especially in view of the growing need to exploit marine resources, HABs and toxic species may represent an increasing risk for human health and economic activities.

Few are the papers reviewing the occurrence of harmful species and/or events at the scale of the whole Mediterranean basin. Fifty years ago, Jacques and Sournia (1978-1979) published a first account of the cases of water discoloration ('eaux rouges') and the species involved. The overview included mainly dinoflagellate blooms, along with a few cases of anoxia but with no evidence of toxic effects in humans or marine fauna in those years when microalgal toxins were still almost unknown. In an overview of nearly twenty years later, cases of PSP and DSP – mainly attributable to *Alexandrium minutum* and *Dinophysis* spp., respectively – were reported from the northern coasts of the basin, along with the records of various potentially toxic or ichthyotoxic dinoflagellates at different sites (Honsell et al., 1995). A subsequent overview of toxic and harmful microalgae covering up to 2009 pointed at the sudden spreading of *Ostreopsis* cf. *ovata* blooms along the rocky Mediterranean shores (Zingone, 2010).

The present overview covers the MS distribution of marine, toxin-producing microalgae, as included in the IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae (Moestrup et al., 2009) and the cases of toxin-related harmful events (Sections 2.1 and 2.2), including direct impact on human health or natural resources or indirect impact to aquaculture industry. In addition, we review non-toxic events that include high biomass harmful algal blooms (HB-HABs) causing seawater discolorations, anoxia or any other damages to the environment or human activities (Section 2.3). Finally, we discuss the trends of HABs in the MS in general and particularly in the Adriatic Sea, which is considered a HAB hotspot (Section 3). The overview is based on information from more than 600 scientific publications and technical reports collected in two curated databases in the Ocean Biogeographic Information System OBIS (Zingone et al., 2022): the MS-HABMAP-OBIS (https://obis.org/), gathering records of toxic species occurrence, and the Harmful Events Database (HAEDAT, http://haedat.iode.org/), collecting information of either toxic or non-toxic events, i.e., cases of intoxications, closures of aquaculture plants, seawater discolorations and mucilages. The present review is a contribution to a first appraisal of the current knowledge of HAB occurrences across the world seas, namely, the Global HAB Status Report, (Hallegraeff et al., 2017; Zingone et al., 2017). The requirement for such an assessment has emerged from the apparent worldwide increase and spreading of HABs and their negative impacts contrasted by the lack of an overview founded on a robust basis of data.

2. HABs in the Mediterranean Sea: toxic species and harmful event distribution

2.1. Toxic species

Of the more than 140 potentially toxic species listed in the IOC-UNESCO taxonomic reference list (Moestrup et al., 2009), 84 have been found in the MS so far: 17 diatoms, 54 dinoflagellates, 3 dictyochophytes, 6 haptophytes, and 4 raphidophytes (Table 3- 1), and some examples in Figure 3-1. These records cover both species actually found to produce toxins in the MS and species known to be toxic from other areas. Given the known variability in toxin production among strains of the same species, non-tested local populations are only ‘potentially toxic’ in most cases, but for brevity they will be referred to as ‘toxic’ in the context of this paper. Sixteen of the toxic species have actually been discovered and described from the MS (Table 3- 2), the first ones (*Prorocentrum lima*, *Dinophysis caudata*, *D. sacculus* and *D. tripos*) in the second half of the 19th century and the most recent ones (*Vulcanodinium rugosum*, *Azadinium dexteroporum*, *Nitzschia bizertensis* and *Ostreopsis fattorussoi*) in the current decade. Some of the HAB species of the MS, such as *D. caudata* and *Chattonella subsalsa*, are widely distributed worldwide while others, including the recently described *N. bizertensis* and *O. fattorussoi*, so far seem to be restricted to specific areas of the MS.

Table 3- 1. Potentially toxic species in the Mediterranean Sea and associated types of syndromes or impacts (see Moestrup et al. (2009) and Lassus et al. (2016) for details). ASP, amnesic shellfish

poisoning; AZP, azaspiracid shellfish poisoning; DSP, diarrhoetic shellfish poisoning; PSP, paralytic shellfish poisoning; CFP, ciguatera fish poisoning. 'Other toxins' include unknown toxins or toxins with poorly known effects.

Bacillariophyceae	
Halamphora coffeaeformis	ASP
Nitzschia bizertensis	ASP
Pseudo-nitzschia australis	ASP
Pseudo-nitzschia brasiliana	ASP
Pseudo-nitzschia caciantha	ASP
Pseudo-nitzschia calliantha	ASP
Pseudo-nitzschia cuspidata	ASP
Pseudo-nitzschia delicatissima	ASP
Pseudo-nitzschia fraudulenta	ASP
Pseudo-nitzschia galaxiae	ASP
Pseudo-nitzschia hasleana	ASP
Pseudo-nitzschia multiseries	ASP
Pseudo-nitzschia multistriata	ASP
Pseudo-nitzschia pseudodelicatissima	ASP
Pseudo-nitzschia pungens[1]	ASP
Pseudo-nitzschia subfraudulenta	ASP
Pseudo-nitzschia subpacifica	ASP
Dictyochophyceae	
Pseudochattonella farcimen	Ichthyotoxicity
Pseudochattonella verruculosa	Ichthyotoxicity
Vicicitus globosus	Ichthyotoxicity
Dinophyceae	
Alexandrium andersonii	PSP
Alexandrium balechii	Ichthyotoxicity
Alexandrium minutum	PSP
Alexandrium ostenfeldii	PSP
Alexandrium pacificum[2]	PSP
Alexandrium pseudogonyaulax	Ichthyotoxicity
Alexandrium tamarense[2]	PSP
Alexandrium taylorii	PSP
Amphidinium carterae	Ichthyotoxicity
Amphidinium klebsii	Ichthyotoxicity
Azadinium dexteroporum	AZP
Azadinium poporum	AZP
Dinophysis acuminata	DSP
Dinophysis acuta	DSP
Dinophysis caudata	DSP
Dinophysis fortii	DSP
Dinophysis infundibulum	DSP
Dinophysis ovum	DSP

Dinophysis sacculus	DSP
Dinophysis tripos	DSP
Fukuyoa paulensis	CFP
Gambierdiscus australes	CFP
Gambierdiscus cf. *belizeanus*	CFP
Gambierdiscus carolinianus	CFP
Gambierdiscus silvae	CFP
Gonyaulax spinifera	Other toxins
Gymnodinium catenatum	PSP
Karenia bicuneiformis	Ichthyotoxicity
Karenia brevis	Ichthyotoxicity
Karenia cristata	Ichthyotoxicity
Karenia longicanalis	Ichthyotoxicity
Karenia mikimotoi	Ichthyotoxicity
Karenia papilionacea	Ichthyotoxicity
Karenia selliformis	Ichthyotoxicity
Karlodinium armiger	Ichthyotoxicity
Karlodinium corsicum	Ichthyotoxicity
Karlodinium veneficum	Ichthyotoxicity
Lingulodinium polyedra	Other toxins
Margalefidinium polykrikoides	Ichthyotoxicity
Ostreopsis fattorussoi	Airborne disease
Ostreopsis cf. *ovata*	Airborne disease
Ostreopsis cf. *siamensis*	Airborne disease
Pfiesteria piscicida	Ichthyotoxicity
Phalacroma mitra	DSP
Phalacroma rotundatum	DSP
Polykrikos hartmannii	Ichthyotoxicity
Prorocentrum borbonicum	Other toxins
Prorocentrum cordatum	Other toxins
Prorocentrum emarginatum	Other toxins
Prorocentrum lima	DSP
Prorocentrum mexicanum	Other toxins?
Prorocentrum rhathymum	DSP
Protoceratium reticulatum	Other toxins
Vulcanodinium rugosum	Other toxins
Haptophyceae	
Chrysochromulina leadbeateri	Ichthyotoxicity
Phaeocystis cf. *globosa*	Other toxins
Prymnesium calathiferum	Ichthyotoxicity
Prymnesium faveolatum	Ichthyotoxicity
Prymnesium parvum	Ichthyotoxicity
Prymnesium polylepis	Ichthyotoxicity
Raphidophyceae	
Chattonella marina[3]	Ichthyotoxicity
Chattonella subsalsa	Ichthyotoxicity

Heterosigma akashiwo	Ichthyotoxicity
Fibrocapsa japonica	Ichthyotoxicity

[1] Including *P. pungens* var. *aveirensis*.

[2] *A. pacificum* (group IV) and *A. tamarense* (group III), following the ribotype group designation in John et al. (2014) and Litaker et al. (2018).

[3] Including *Chattonella marina* var. *antiqua*.

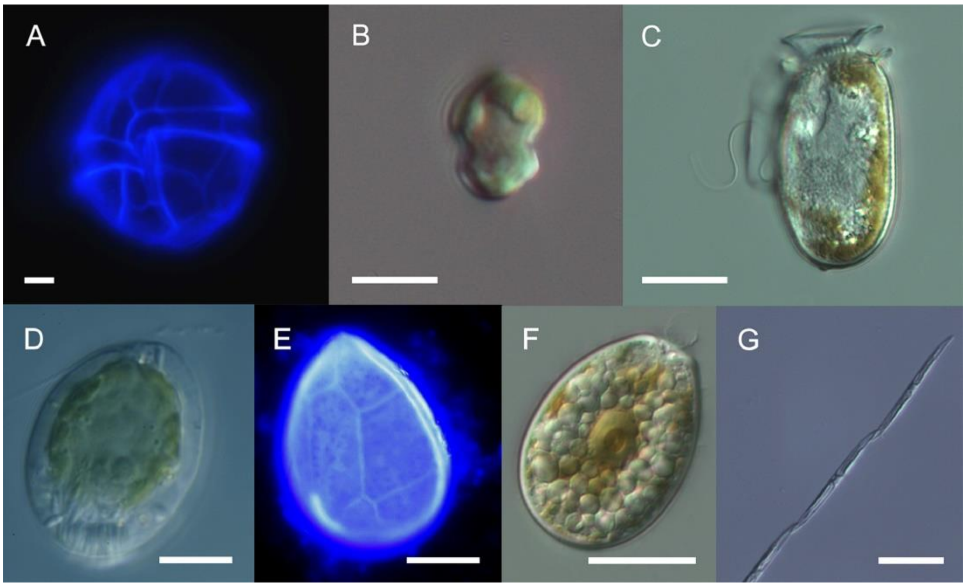

Figure 3- 1. Examples of toxic species from the Mediterranean Sea. A) *Alexandrium minutum* stained with calcofluor. B) *Azadinium dexteroporum*. C) *Dinophysis sacculus*. D) *Fibrocapsa japonica*. E) *Ostreopsis fattorussoi* stained with calcofluor (courtesy of S. Accoroni). F) *Prorocentrum lima*. G) *Pseudo-nitzschia multistriata*. Scale bars in A and B: 5 µm; in C, D, E, F and G: 20 µm.

Table 3- 2. Potentially toxic species described from the Mediterranean Sea.

Species name	Described in	Described as	Type locality
Alexandrium minutum Halim	Halim (1960a)		Harbour of Alexandria, Egypt
Alexandrium pseudogonyaulax (Biecheler) Horiguchi ex K.Yuki & Y.Fukuyo	Biecheler (1952)	*Goniodoma pseudogonyaulax*	Thau Lagoon, Gulf of Lion, France

Azadinium dexteroporum Percopo & Zingone	Percopo et al. (2013)		Gulf of Naples, Italy
Chattonella subsalsa Biecheler*	Biecheler (1936)		Saltern of Villeroy, Sète, France
Dinophysis caudata Kent	Kent (1881)		Nearby Fano, Marche Region, Italy
Dinophysis fortii Pavill.	Pavillard (1923)		Thau Lagoon and/or Sète harbour, France
Dinophysis infundibulum J.Schiller	Schiller (1928)		Southern Adriatic Sea
Dinophysis sacculus F.Stein	Stein (1883)		Kvarner Gulf, Croatia
Dinophysis tripos Gourret	Gourret (1883)		South of Ratonneau, Gulf of Marseille, France
Karlodinium armiger Bergholtz, Daugbjerg & Moestrup	(Bergholtz et al., 2006)		Alfacs Bay, Catalonia, Spain
Karlodinium corsicum (Paulmier, Berland, Billard & Nézan) Siano & Zingone	Paulmier et al. (1995)	*Gyrodinium corsicum*	Diana Lagoon, Corse, France
Nitzschia bizertensis Bouchouicha-Smida, Lundholm, Hlaili & Mabrouk	Bouchouicha-Smida et al. (2014)		Bizerte Lagoon, Tunisia

Ostreopsis fattorussoi Accoroni, Romagnoli & Totti	Accoroni et al. (2016)		Batroun, Lebanon
Prorocentrum lima (Ehrenb.) F.Stein	Ehrenberg (1860)	*Cryptomonas lima*	Sorrento, Gulf of Naples, Italy
Prymnesium faveolatum Fresnel	Fresnel et al. (2001)		Beach of Roquebrun, Cap Martin, France
Vulcanodinium rugosum Nézan & Chomérat**	Nezan and Chomerat (2011)		Ingril Lagoon, France
* A second, distinct genotype also discovered in Mediterranean waters (Klopper et al., 2013).			
** First report in Rhodes et al. (2010) from New Zealand.			

The discovery of potentially toxic species in the MS has undergone an evident escalation over the years (Figure 3- 2), from the first descriptions of more than a century before the discovery of their toxicity to the rapid increase after the 1960s and the most recent findings. Information on their distribution has also markedly increased along with the intensification of monitoring operations and studies on planktonic and benthic microalgae (e.g., Aligizaki et al. (2009); Balkis and Tas (2016); Fernández et al. (2019); Pistocchi et al. (2012); Zingone et al. (2006)) and of their resting stages in the sediments (Bravo et al., 2006; Satta et al., 2013) or sediment traps (Montresor et al., 1998). Yet the actual range of most toxic species in the MS is far from being known. Indeed, the identification of some of the most represented genera in the MS, such as *Alexandrium*, *Karenia*, *Karlodinium* and *Pseudo-nitzschia*, as well as of many other flagellates, is quite problematic. In many cases the observation of live material or methods more complex than light microscopy are needed. Cryptic diversity discovered in many microalgal taxa over the last decades also concerns several harmful genera and species, which have undergone careful taxonomic investigations more than other non-toxic taxa. This trend has led to the discovery of non-toxic taxa morphologically similar to toxic ones, such as several species in the *P. delicatissima* and *P. pseudodelicatissima* species-complexes (Bates et al., 2018), the non-toxic *A. tamutum* hardly distinguishable from *A. minutum* (Figure. 1A, Montresor et al. (2004)) and the non-toxic, chain-forming *Gymnodinium impudicum* (as *Gyrodinium impudicum*, Fraga et al. (1995)) which was misidentified as *Gymnodinium catenatum* in studies predating its discovery (e.g., Carrada et al. (1991)). Recent studies coupling detailed morphological investigations with the analysis of different molecular markers and toxin production have attempted to clarify species identity within the *Alexandrium tamarense*-species complex (John et al., 2014; Litaker et al., 2018). The case of *Chattonella subsalsa* is interesting because, based on several molecular markers, two different genotypes with different geographic distributions exist for the species (Klopper et al., 2013). All these taxonomic insights have invalidated many previous identifications of presumed toxic taxa, as detailed in the following sections. In recent years, information on the presence of toxic species is also gathered through molecular identification of environmental DNA samples (e-DNA metabarcoding), which may give relevant information on the presence and seasonality of cryptic or rare species (Dzhembekova et al., 2017; Grzebyk et al., 2017; Ruggiero et al., 2015). Nonetheless, new findings of species through molecular methods should always be confirmed by morphological studies.

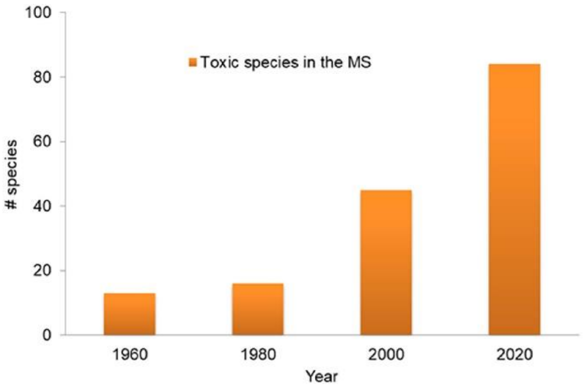

Figure 3- 2. Cumulative numbers of known toxic species in the Mediterranean Sea in different years.

Some of the toxic species of the MS have been suspected to be non-indigenous species (NIS), i.e., introduced outside their natural past or present distribution. The main possible NIS in the MS are *Pseudo-nitzschia multistriata*, *Alexandrium pacificum* and *Ostreopsis* cf. *ovata*. The first MS record of *Pseudo-nitzschia multistriata*, a chain-forming diatom having a distinctive sigmoid shape (Fig. 1G), was in 1992 in the Gulf of Naples, where phytoplankton have been intensively studied since the beginning of the 1980s. The species has shown an increasing trend afterwards in the same area (D'Alelio et al., 2010) and has subsequently been found in Spanish (Quijano-Scheggia et al., 2008), Greek (Moschandreou and Nikolaidis, 2010), Tunisian (Sahraoui et al., 2011) and Moroccan waters (Rijal Leblad et al., 2013) and in the Adriatic Sea (Pistocchi et al., 2012; Turk Dermastia et al., 2020). The chain-forming dinoflagellate *Alexandrium pacificum* (as *A. catenella*) was found for the first time in low density in 1983 along the Spanish coast (Margalef and Estrada, 1987). In the following years, *A. pacificum* formed blooms on the Spanish coast (Gomis et al., 1996; Vila et al., 2000) and in the Thau Lagoon (as *A. tamarense*/*catenella*, (Abadie et al., 1999; Lilly et al., 2002)). Subsequently it was progressively found eastward along the Italian (Lugliè et al., 2003; Luglie et al., 2017; Satta et al., 2013), Algerian (Frehi et al., 2007) and Tunisian coasts (Fertouna-Bellakhal et al., 2015; Turki et al., 2007), whereas it is still unrecorded in the rest of the MS. The benthic dinoflagellate *Ostreopsis* cf. *ovata* showed a sudden emergence in the MS at the end of the last century (see Section 2.2.4). A much higher genetic variability and several cryptic species characterize this taxon along the Japanese coasts compared to the Mediterranean-Atlantic area (Penna et al., 2012; Sato et al., 2011) where genetic differences are seen only at the population level with AFLP markers (Italiano et al., 2012). This situation suggests a relatively recent radiation of the species in the latter area and given the lack of hydrographic links between the two regions, a possible man-mediated transport, although it is impossible to establish when this occurred (Sato et al., 2011). In lack of type material, or material from the type locality, it has not been established which of the numerous morphologically similar taxa corresponds to *Ostreopsis ovata*. Therefore, these taxa should be referred to as *O.* cf. *ovata* (Penna et al., 2010; Sato et al., 2011). Benthic *Gambierdiscus* and *Fukuyoa* species are also a novelty in the MS, and their distribution, presently limited at the two ends of the basin, hints at a possible recent introduction from both the Atlantic and the Red Sea.

2.2. Toxic events

2.2.1. Diarrhoetic shellfish poisoning (DSP)

DSP toxins in mollusks represent the most frequently reported cases of seafood contamination in the MS. Eight toxic species of the genus *Dinophysis*, plus two of the genus *Phalacroma* (Table 3- 1), have been observed along the Mediterranean coasts (Figure 3- 3). *Dinophysis caudata* and *D. sacculus* (Figure 3- 1), the most frequently reported species, were both described from the MS more than one century ago (Kent, 1881; Stein, 1883), but risks for human health have first been recognised only in the 1980s in the Gulf of Lion (Belin et al., 1995). In the northern Adriatic Sea, DSP toxicity events have occurred on both the western and eastern side, often causing the closure of shellfish farms (Bernardi Aubry et al., 2000; Boni et al., 1992; Boni et al., 1993; Della Loggia et al., 1993; France and Mozetič, 2006; Marasovic et al., 2007; Nincevic-Gladan et al., 2008; Orhanovic et al., 1996; Sedmak and Fanuko, 1991). In the period 1989–2018, such closures occurred regularly along the Slovenian coast (northern Adriatic) with an exceptionally long period from May 2010 to March 2011 in which relatively high *Dinophysis* abundances were recorded (around 2000 cells·L^{-1} of *D. fortii*, (Francé et al., 2018)). These high abundances, never recorded again, were related to long-lasting low salinity and extremely high temperatures in June – July surface waters (<30 °C) causing a marked water column stratification (Francé et al., 2018).

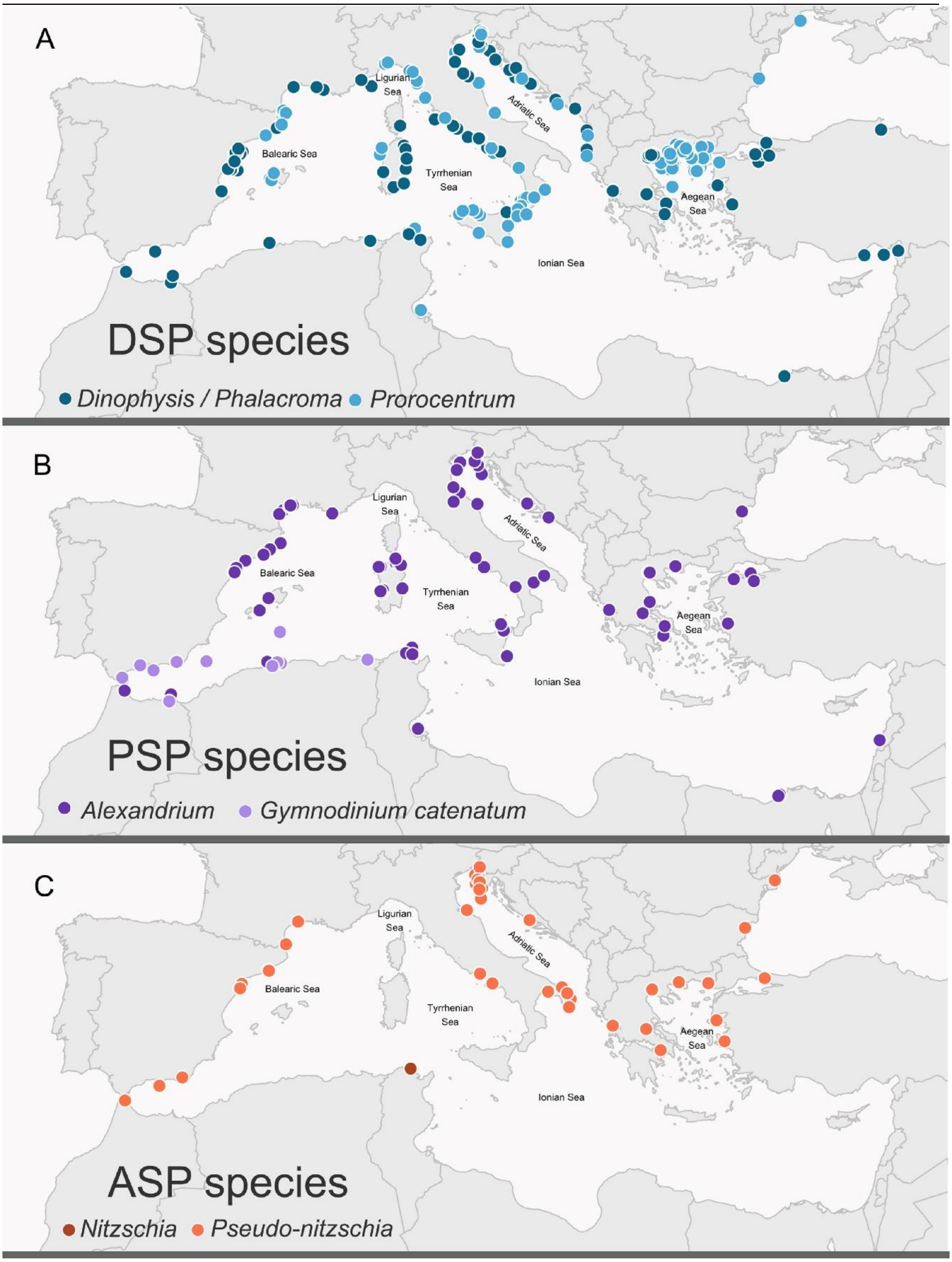

Figure 3- 3. Geographic range of potentially toxic species in the Mediterranean Sea. Distribution of species known to produce toxins related to: A) Diarrhoetic shellfish poisoning (DSP), *Dinophysis* spp. and the benthic species *Prorocentrum lima* and *P. rhathymum*. B) Paralytic shellfish poisoning (PSP), *Alexandrium* spp. and *Gymnodinium catenatum*. C) Amnesic shellfish poisoning (ASP), *Pseudo-nitzschia* spp. and *Nitzschia bizertensis*. For the genera *Dinophysis*, *Pseudo-nitzschia* and *Alexandrium*, which include both toxic and non-toxic species, the maps represent only toxic species and, in case of

cryptic or problematic species, only the records validated by electron microscopy, molecular methods and/or toxin production.

High levels of okadaic acid (OA) and/or dinophysistoxin (DTX) in several instances also led to halt shellfish harvesting along the French (Belin et al., 2021) and Spanish coasts of the MS (Fernández et al., 2019; García-Altares et al., 2016). Recurrent toxic *Dinophysis* blooms have been recorded in the Thermaikos Gulf (Greece, North Aegean Sea) since 2000, when they caused great economic losses (€ 5 million) to aquaculture (Koukaras and Nikolaidis, 2004). More occasionally, high levels of DSP toxins have been reported from the eastern Mediterranean (Bazzoni et al., 2018; Orhanovic et al., 1996) and Tunisian waters (Armi et al., 2012).

Nonetheless, there have been just a few cases of DSP diagnoses in humans, in the Adriatic ((Boni et al., 1992) and Tyrrhenian Seas (Lugliè et al., 2011), and two major accidents. One occurred in 2000, when 200 people were hospitalized following the above-mentioned *Dinophysis* bloom in the Thermaikos Gulf (Koukaras and Nikolaidis, 2004). The other happened in 2010 in Piemonte (north-western Italy), with more than 150 people harmed by the consumption of toxic mussels from the northern Adriatic Sea (Pistocchi et al., 2012).

Other DSP producers widely distributed in the MS are two benthic species of the genus *Prorocentrum* (Figure 3- 3), *P. lima* (Figure 3- 1) and *P. rhathymum*, but no toxicity events have been related with their presence.

2.2.2. Paralytic shellfish poisoning (PSP)

PSP events in the MS are related to toxins produced by species of the genus *Alexandrium* and by *Gymnodinium catenatum*. Of the six *Alexandrium* species known to produce PSP toxins found in the MS, *A. minutum*, the type species of the genus (Figure 3- 1), and *A. pacificum* (as *A. catenella* in records before 2014) are the most commonly reported ones (Table 3- 1, Figure 3- 3F). In some cases, these species have reached high densities (up to 107 cells·L^{-1}) causing seawater discolorations. *Alexandrium taylorii* has also caused discolorations at several Spanish and Italian touristic places (Section 2.3.1, Table 3S- 1) but no toxicity has ever been found in MS populations of this species.

Reports of PSP events initially associated with *A. tamarense* (Abadie et al., 1999; Boni et al., 1983; Honsell et al., 1992), a species that should not produce saxitoxins (John et al., 2014), were later reinterpreted and attributed to *A. minutum* (Pistocchi et al., 2012) or *A. pacificum* (Lilly et al., 2002). However, one strain of *A. tamarense* from Sardinian coasts has recently been found to be toxic (Luglie et al., 2017). Since the first observations of massive natural fish mortalities in Egypt (Labib and Halim, 1995; Zaghloul and Halim, 1992), *A. minutum* produced toxic blooms with consequent ban of both fishing and shellfish harvesting in Morocco (Tahri-Joutei et al., 2003), Spain (Delgado et al., 1990; Forteza et al., 1998), France (Belin et al., 2021) and Italy (Honsell et al., 1996). After

2000, only a few cases of shellfish farm closures attributed to *A. minutum* have been reported in northern Sardinia (Italy; (Lugliè et al., 2011)), Catalonia (Spain; (Bravo et al., 2008; Sampedro i Roig, 2018; Vila et al., 2005) and southern France coasts (Belin et al., 2021). Because of a very similar non-toxic species discovered in the MS, *A. tamutum*, the identification of *A. minutum* can be problematic and should be confirmed by molecular or toxin analyses. *Alexandrium pacificum* was responsible for toxic blooms along the Catalan coast (Bravo et al., 2008), in the Thau Lagoon (Abadie et al., 1999), in Sardinia (Lugliè et al., 2011) and Sicily (Dell'Aversano et al., 2019), at times causing shellfish harvesting closures (Bravo et al., 2008; Vila et al., 2000).

Alexandrium andersonii and *A. ostenfeldii* are much less frequently recorded and possibly overlooked or misidentified in plankton studies. At times their presence has been traced as resting stages (e.g., (Bravo et al., 2006; Montresor et al., 1998; Satta et al., 2013)). Two other *Alexandrium* species recorded in the MS, *A. balechii* and *A. pseudogonyaulax*, do not produce PSP toxins but are considered potentially ichthyotoxic.

Gymnodinium catenatum was first reported in southern Spain in 1987 (Bravo et al., 1989). The worst, and apparently unique, fatal case of human intoxication in the whole Mediterranean was due to a bloom of this species that caused 4 deaths and the hospitalization of 23 people in Morocco in 1994 (Tagmouti-Talha et al., 1996). Shellfish harvesting ban due to high concentrations of *G. catenatum* have been frequent in Andalusia (Spain) during the last 3 decades (HAEDAT). Several records of this species in the central and eastern MS should be considered with caution because of possible misidentification of *G. impudicum* (Gómez, 2003).

2.2.3. Amnesic shellfish poisoning (ASP)

Fifteen of the 26 *Pseudo-nitzschia* species known to produce domoic acid (DA) have been found so far in the MS. Species-level identification is problematic in light microscopy and often requires the use of electron microscopy and/or molecular markers. It follows that in most publications only the genus is reported, or taxa are clustered into two 'groups', only distinguishing the thin (*P. delicatissima*-group) and the thicker morphotypes (*P. seriata*-group). In the last decades, potentially toxic *Pseudo-nitzschia* species have been identified properly from several locations of the MS (Figure 3- 3) where the presence of the cold-water species *Pseudo-nitzschia seriata*, often reported in old studies, has never been confirmed.

Seasonal blooms of *Pseudo-nitzschia* spp., at times including toxic ones, occur all along Mediterranean coasts (Figure 3S- 1), with abundances up to several million cells·L^{-1} (e.g., (Cabrini et al., 2012; Caroppo et al., 2005; Cerino et al., 2005; Ljubešić et al., 2011; Marić et al., 2011; Quijano-Scheggia et al., 2008; Quiroga, 2006; Ruggiero et al., 2015; Tas and Lundholm, 2017; Totti et al., 2019b). Nevertheless, the detection of DA has caused the closure of aquaculture plants only in a limited number of cases (4 % of toxicity events in HAEDAT) in southern Spain (HAEDAT) and

France (Amzil et al., 2001), whereas DA values below the regulatory limit have occasionally been found in shellfish from the Adriatic Sea (Arapov et al., 2016; Ciminiello et al., 2005; Ujević et al., 2010), Greece (Kaniou-Grigoriadou et al., 2005), and in 65 % of 180 mussel samples from mid-Tyrrhenian waters (Rossi et al., 2016). In a few cases, the presence of DA in bivalves was related to a specific taxon, i.e., *P. calliantha* along the Croatian coast (Marić et al., 2011) and in the Gulf of Trieste (Honsell et al., 2008) and *P. brasiliana* in the Bizerte Lagoon in Tunisia (Sahraoui et al., 2011).

Nitzschia bizertensis, described from the Bizerte Lagoon (Tunisia), is one of the two *Nitzschia* species known to produce domoic acid. At least in one case, the presence of this species was related to the detection of domoic acid in mussels (Bouchouicha-Smida et al., 2014). Less clear is the toxicity and the distribution of the other benthic diatom *Halamphora coffeaeformis*.

2.2.4. **Ostreopsis** *and species responsible of ciguatera fish poisoning (CFP)*

The benthic dinoflagellate *Ostreopsis* cf. *ovata* produces ovatoxins, which are palytoxins-like molecules that can intoxicate humans by inhalation or ingestion of contaminated seafood. The species was first detected in the MS in the plankton of Villefranche-sur-Mer (France) after a strong mistral wind event in 1972 (Max Taylor, pers. comm.), when it was identified with the name of the only species known at that time, *O. siamensis*. The presence of the species was then documented from the coasts of Lebanon in 1980 (Abboud-Abi Saab, 1989) and central Italy in 1986 (Zingone in Tognetto et al. (1995)). Around the 2000s, monitoring programs implemented following a series of harmful events (see below) made it evident that *Ostreopsis* species were growing all along the rocky shores of the northern MS (Figure 3- 4) in summer/autumn, thriving as epiphyte on macroalgae or epibionthic on a number of benthic substrata, with concentrations up to 107 cells·g^{-1} fresh weight of macroalgal thalli (Mangialajo et al., 2011). At lower concentrations, *Ostreopsis* spp. were also found along the northern African coasts (Ben Gharbia et al., 2019; Illoul et al., 2012). Of the three species so far identified in the MS, the most common and widespread is *O.* cf. *ovata*, whereas *O.* cf. *siamensis* and *O. fattorussoi* have a much more restricted distribution (Figure 3- 1). An interesting aspect of the annual dynamics of *Ostreopsis* species is the rather repetitive patterns of summer and/or autumn peaks, with timing that vary from place to place and is scarcely related to temperature or to other obvious environmental parameters (Accoroni et al., 2016; Aligizaki and Nikolaidis, 2006; Zingone, 2010).

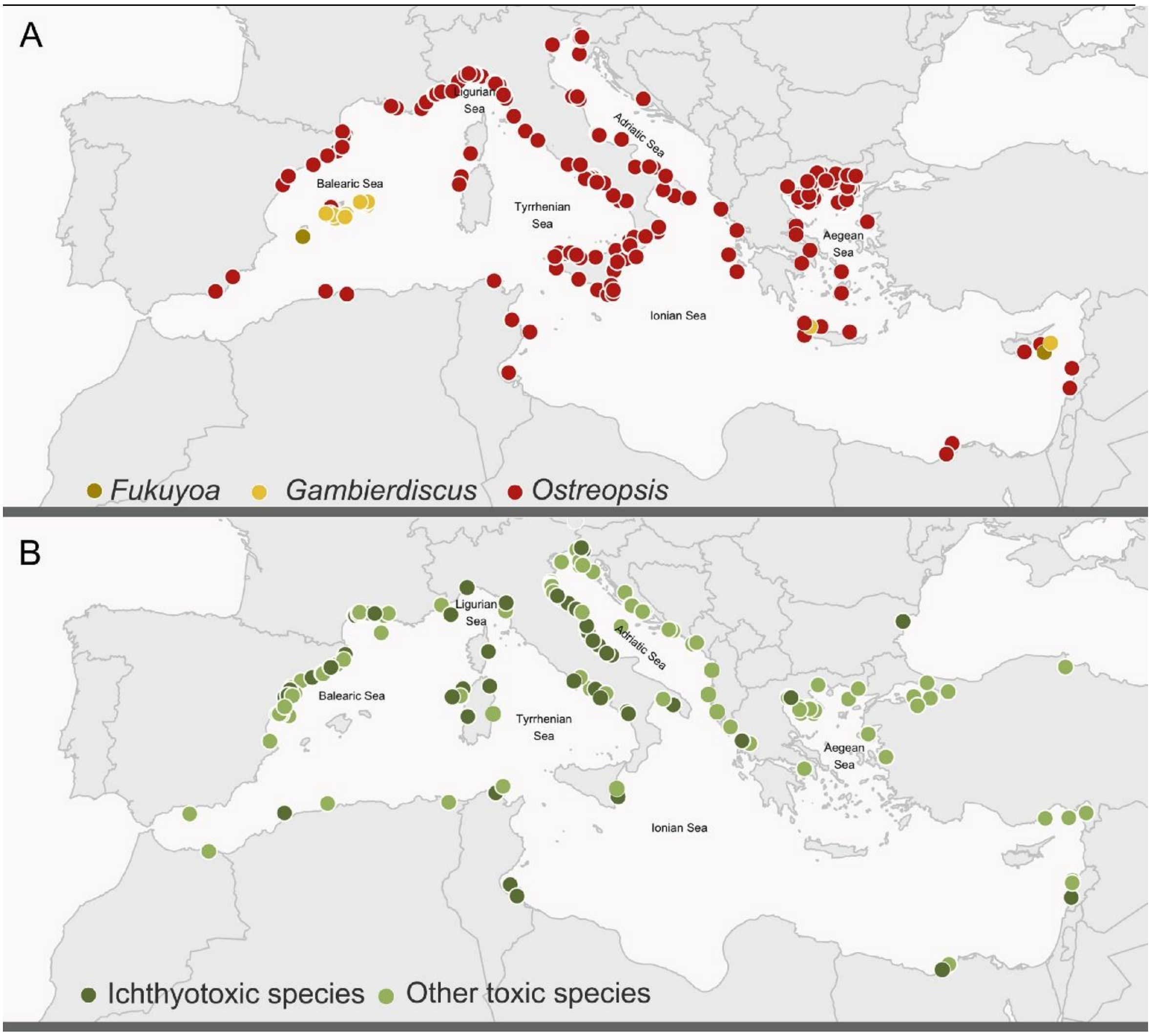

Figure 3- 4. Fig. 4. Geographic range of potentially toxic species in the Mediterranean Sea. A) *Ostreopsis* spp. (mostly *O.* cf. *ovata*) and other benthic dinoflagellate species related to the ciguatera fish poisoning (CFP). B) Species producing ichthyotoxins (*Alexandrium pseudogonyaulax*, *Karenia* spp., *Karlodinium* spp., *Chattonella* spp., *Vicicitus globosus*, *Prymnesium* spp., etc.) and other toxins. The latter include mainly a few widespread dinoflagellate species that produce yessotoxins (*Lingulodinium polyedra*, *Gonyaulax spinifera* and *Protoceratium reticulatum*), but also other dinoflagellates producing azaspiracids (*Azadinum* spp.), pinnatoxins (*Vulcanodinium rugosum*) and other toxins with poorly known effects (e.g., *Prorocentrum* spp.). See Table 1 for a complete list.

First problems caused by *Ostreopsis* in the MS were fish and invertebrate kills in 1998 along the coasts of Tuscany (northern Tyrrhenian Sea) (Sansoni et al., 2003; Simoni et al., 2003). Some years later (2002) more than 200 people coming from the beach of the city of Genoa (Ligurian Sea) were hospitalized with fever, red eyes and wheeze (Ciminiello et al., 2006). The only known problems caused by benthic microalgae at that time were those related to ciguatera fish poisoning (CFP) in subtropical areas, whereas cases of toxic aerosol were only known for planktonic *Karenia brevis* blooms in the Gulf of Mexico. In those years, similar human health problems and dermatitis cases were reported from the Catalonia and Balearic Islands (Vila et al., 2008), French (Cohu et al., 2013) and Algerian coasts (Illoul et al., 2012), and are still reported nowadays at several MS places (e.g., Croatian coast, (Ninčević Gladan et al., 2019). Both the presence of toxins in the aerosol (Ciminiello et al., 2014) and toxicological data on the effects of inhalation exposure in mice (Poli et al., 2018) support a link between *Ostreopsis* toxins and the respiratory symptoms reported during blooms. However, those health problems do not occur during all phases of a bloom (Vila et al., 2016) and are quite sporadic compared to the widespread and often massive presence of the suspected causative species.

The presence of *Ostreopsis* toxins in marine animals used as food and their impacts on the animal health are relevant for their sanitary implications, which are still controversial (Tubaro et al., 2011). Apparently healthy organisms (e.g., mussels and sea urchins) during *Ostreopsis* blooms can accumulate fairly large amount of toxins (Aligizaki et al. (2008); E. Fattorusso & V. Soprano, pers. comm.), but macroscopic damages have been reported for various benthic organisms in the MS (Accoroni et al., 2016; Sansoni et al., 2003; Simoni et al., 2003) and elsewhere (Shears and Ross, 2009). In mussels, *Ostreopsis* can induce important and not completely reversible ultrastructural damages (Carella et al., 2015) and immunological, histological and oxidative responses (Gorbi et al., 2013) while in sea urchins *Ostreopsis* blooms affect reproduction and offspring health (Migliaccio et al., 2016).

Four species of the dinoflagellate genus *Gambierdiscus*, which can produce CFP toxins, have recently been found in the MS. *Gambierdiscus australes*, *G.* cf. *belizeanus*, *G. carolinianus*, *G. silvae* and some unidentified *Gambierdiscus* spp., have been reported from the Balearic Islands (Tudó et al., 2018), Greece and Cyprus (Aligizaki et al., 2018; Aligizaki and Nikolaidis, 2008; Holland et al., 2013; Tudó et al., 2018), with the highest diversity in Crete. *Fukuyoa paulensis* also has been found in the Balearic Islands (Laza-Martínez et al., 2016) and Cyprus (Tudó et al., 2018). Yet CFP cases are not known in the MS countries with the exception of a suspected case of ciguatoxins in rabbitfish (*Siganus rivolutus*) reported from Israeli coasts (Bentur and Spanier, 2007).

2.2.5. Azaspiracid shellfish poisoning (AZP)

The toxins azaspiracids (AZAs), produced by a number of dinoflagellate species of the genera *Azadinium* and *Amphidoma*, and the human syndrome they can cause, AZP, have been discovered at the beginning of this century (James et al., 2002). Subsequently AZAs have been reported in shellfish from numerous sites, including the MS (Bacchiocchi et al., 2015). A new species described from the MS, *A. dexteroporum* ((Percopo et al. (2013); Figure 3- 1), produces a whole suite of AZAs that can cause direct harm to molluscs (Giuliani et al., 2019; Rossi et al., 2017). Another toxic *Azadinium*, *A. poporum*, has been found in Greek waters (Luo et al., 2018) but no impacts related to AZAs have been reported so far.

2.2.6. Ichthyotoxicity

About half of the potentially toxic MS species produce a variety of toxins that differ from those related to the syndromes mentioned in the previous sections. Of these, the majority (29 species, Table 3- 1) produce substances that have been associated with fish and/or shellfish kills. With a few exceptions, species in this list are unarmoured dinoflagellates, e.g., *Karenia* and *Karlodinium*, and other flagellates belonging to the prymnesiophytes, raphidophytes and dictyochophytes, which are all hardly identifiable in fixed material under the light microscope, and hence are overlooked in most monitoring and ecological investigations. The large majority of the information on the presence of these ichthyotoxic species (Figure 3- 4) comes from fish mortality events, mainly located near fish-farming plants, in which the identification of the culprit became necessary.

The few fish mortality events in the MS known before 1975 were related to HB-HABs of non-ichthyotoxic species causing anoxia in bottom waters (see Section 2.3.1) rather than to ichthyotoxic species (Jacques and Sournia, 1978-1979). In the subsequent years, fish kills by ichthyotoxic species were reported sporadically from Catalan coasts, Spain (Garcés et al., 1999a), caused by *Karlodinium* spp., and Sardinia (Italy), caused by *Chattonella subsalsa* (Stacca et al., 2016). Occasional fish mortality events were related to *Prymnesium* spp., in the Ebro Delta (Spain, Comín and Ferrer (1978)) and in a Tuscany lagoon (Italy, Mattioli and Simoni (1999)), *Karenia selliformis* in the Gulf of Gabes (Tunisia, (Feki et al., 2013; Romdhane et al., 1998)) and *Karenia brevis* and *Pseudochattonella* cf. *verruculosa* in Greece (Ignatiades and Gotsis-Skretas, 2010). In other cases, fish kills occurred during blooms of species toxic to humans, like in Egypt in 1987 (Labib and Halim, 1995; Zaghloul and Halim, 1992) where *Alexandrium minutum* was the culprit. No fish or shellfish kill accidents in the MS have ever been associated with blooms of two potentially ichthyotoxic *Alexandrium* species, *A. balechii* and *A. pseudogonyaulax*.

Benthic cyanobacteria are poorly investigated in Mediterranean waters, but blooms of filamentous cyanobacteria have been the cause of massive fish mortalities in Alexandria waters (Egypt) during spring 2005 (Ismael, 2012).

2.2.7. Other toxins

The dinoflagellates *Gonyaulax spinifera*, *Lingulodinium polyedra* and *Protoceratium reticulatum*, which are quite widespread in the MS (Figure 3- 4), produce yessotoxins (YTX). These substances were initially associated to DSP because their presence gives similar positive results in mouse bioassay, but they are not considered toxic to humans (Tubaro et al., 2010). However, YTXs caused economic impacts in 2002, 2004 and 2007, when mussel harvesting was halted for a long time (average closure 153 days) in the north-western Adriatic Sea (Poletti et al., 2008).

Vulcanodinium rugosum produces pinnatoxins (Nezan and Chomerat, 2011; Rhodes et al., 2010) a neurotoxin that has lethal effects on sea urchin larvae, oysters and *Artemia*. Currently there are no problems related to this species, while toxic effects on humans are not known.

2.3. Non-toxic events

Independent from toxin production, all microalgae may exert a negative impact when they reach a high biomass producing seawater discolorations, mucilages or anoxia in bottom waters (Zingone and Enevoldsen, 2000). Although several microalgal species are frequently associated with these HB-HABs, as detailed in the next sections, the number of species that may cause harm with no specific toxin production is in theory unlimited, and can vary from place to place. For this reason, it is not possible to define a global or regional list of non-toxic harmful microalgae. In addition to HB-HAB-formers, some non-toxic species, mainly diatoms, may cause mechanical harm to invertebrates' gills (Bell, 1961), but no information on such events is available for the MS. In case of fish or invertebrate kills, at time it is hard to discern whether the cause has been anoxia, toxic substances or mechanical damages. In many cases, species known to produce toxins may produce non-toxic HB-HABs, which have no impact on human or marine fauna health but important consequences for tourism. For all these reasons, the boundaries between events described in the previous and next sections cannot always be well defined.

2.3.1. Discolorations

In the MS, discoloration or anoxia have frequently been caused by unarmoured dinoflagellates either toxic (e.g., *Margalefidinium polykrikoides*) or non-toxic (e.g., *Noctiluca scintillans*), but also by numerous armored dinoflagellates, diatoms, prasinophytes, prymnesiophytes and raphidophytes (Table 3S- 1). Changes of seawater colour caused by HB-HABs (Figure 3- 5) have been noticed since the first half of the XX century in both lagoons and coastal sites, where they were given several names (purga de mar, punti verdi) before the one of red tides gained popularity. The oldest records include discolorations caused by *Chattonella subsalsa* in 1956 in the Algiers harbour (Hollande and Enjumet, 1957), *Alexandrium minutum* in 1957 in the Alexandria harbour (Halim, 1960a) and *Prorocentrum cordatum* in the Gulf of Naples in September 1962 (Yamazi, 1964).

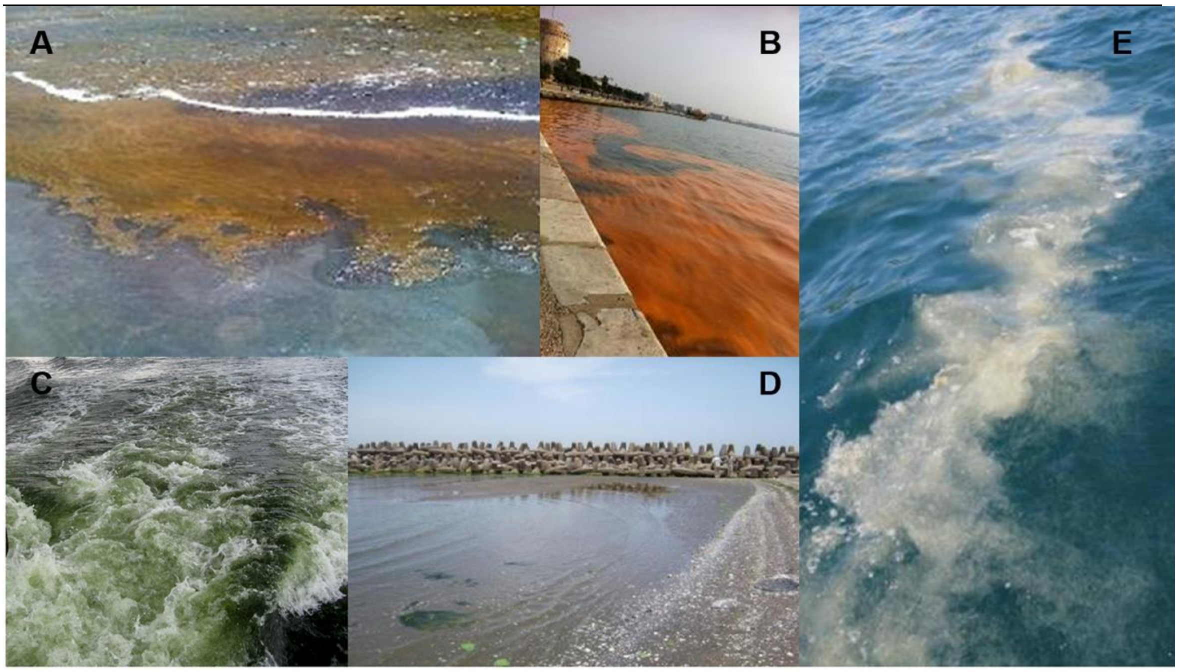

Figure 3- 5. A) Mat of *Oscillatoria acutissima* in the Eastern Harbour of Alexandria (Egypt). B) Bloom of *Noctiluca scintillans* in Thermaikos Gulf (Thessaloniki, Greece). C) Discoloration caused by *Euglena viridis* in the Golden Horn Estuary (Sea of Marmara, Turkey). D) Shellfish mortality in Ras El-Bar (Egypt) in 2011 due to the proliferation of *N. scintillans* and consequent oxygen depletion. E) Pelagic mucilages in the Gulf of Naples (Italy).

Different dinoflagellates (e.g., *Alexandrium* spp., *Noctiluca scintillans*, *Karlodinium* spp.), raphidophytes (*Chattonella subsalsa* and *Fibrocapsa japonica*, Figure 3- 1) and chlorophytes (*Tetraselmis wettsteinii* and *Pyramimonas* spp.) occasionally produced discoloration (Table 3S- 1, Figure 3- 5), which in some cases were also associated with fish kills and/or massive death of marine invertebrates caused by anoxic conditions (e.g., (Arzul et al., 1994; Garcés et al., 1999a; Halim and Labib, 1996)). A couple of such cases of fish mortality events attributed to anoxia were already reported in the review by Jacques and Sournia (1978-1979): in Izmir Bay (Nümann, 1955, in Jacques and Sournia (1978-1979)) and in the Adriatic Sea (Froglia, 1970; Piccinetti and Manfrin, 1969), during blooms of *Gymnodinium* sp. and *Protoperidinium depressum*, respectively.

Discolorations were particularly frequent in the northern Adriatic Sea in summer in the 1970–‘80s, when dinoflagellate blooms (e.g., *Lingulodinium polyedra*, *Alexandrium mediterraneum* and *Lepidodinium chlorophorum*) turned the sea into various colours (Boni (1983), Table 3S- 1), at times extending offshore as in the case of *N. scintillans* in 1980 (Fonda Umani et al., 2004) and *L. chlorophorum* in 1984 (Artegiani et al., 1985). Some summer blooms were caused by diatoms (e.g., *Skeletonema marinoi* and *Chaetoceros* spp.), particularly after intense freshwater inputs (Boni, 1983; Regione Emilia-Romagna, 1982-2018). Over the last decades blooms of *F. japonica* (Figure 3- 1) became common in late summer (Cucchiari et al., 2008) in shallow coastal waters where they lasted up to 20–40 days. Along the eastern Adriatic coast, 'red tides' were limited to eutrophicated semi-enclosed bays (Marasović et al., 1991) or to unusual phenomena such as bloom of the silicoflagellate *Octactis* (formerly *Distephanus*) *speculum* in summer 1983 in bottom waters in the Gulf of Trieste, causing anoxia (Fanuko, 1989). An increasing number of discolorations have been observed over two decades in the Golden Horn Estuary of the Sea of Marmara (Taş et al., 2016). An unusual bloom of the coccolithophore *Holococcolithophora sphaeroidea* (as *Calyptrosphaera sphaeroidea*) caused a white-green-turquoise discoloration in a vast area off the Tarragona harbour (Spain, Cros et al. (2002)). The most recent event has been a long-lasting bloom of *Margalefidinium* cf. *polykrikoides* that produced a yellow brownish discoloration in a touristic area of the Ionian Sea (Italy) in July-August 2018, recurring in the same place in summer 2019 (Roselli et al., 2020).

In summer, discolorations can be a serious problem along Mediterranean beaches where they have an impact on tourism and recreational use of the sea. This is the case of the recurrent *Alexandrium taylorii* blooms along the Sicilian and Sardinian coasts (Italy) and in the Balearic Islands (Spain) (e.g., (Basterretxea et al., 2005; Giacobbe et al., 2007; Sampedro i Roig, 2018; Satta et al., 2010)).

2.3.2. Mucilages

In the MS, a number of cases of mucilaginous aggregate formation related to microalgal growth have been described, the most conspicuous of which occurred in the northern Adriatic Sea in the 1990s. Mucilaginous macroaggregates represent the last stage of aggregation of organic matter, mainly refractory polysaccharides derived from phytoplankton exudates (Myklestad, 1995) and/or from bacterial capsular material (Stoderegger and Herndl, 1998) whose hydrolysis cannot be sustained by phosphorus-limited bacteria (Danovaro et al., 2005). Whereas marine snow (aggregates of 0.5–1 cm diameter) is common in all the oceans (Simon et al., 2002), the mucilage event in the northern Adriatic Sea was unique in that those aggregates covered hundred square kilometres of both coastal and offshore areas. The formation of larger aggregates was favored by the strong stratification of the water column and reduced circulation that retained freshwater in the northern Adriatic basin (Russo et al., 2005). The direct responsible of the phenomenon were often thought to be the most abundant phytoplankton species in the aggregates, such as *Cylindrotheca closterium* (Revelante and Gilmartin, 1991) and *Gonyaulax fragilis* (Pompei et al., 2003), both capable to produce large amounts of refractory polysaccharides (Pistocchi et al., 2005; Urbani et al., 2005). In fact, phytoplankton communities associated with mucilage aggregates largely vary, depending on sampling area and period (Totti et al. (2005), and references therein), while the aggregates represent a self-sustained microcosm hosting a rich microorganism community (Simon et al., 2002).

Pelagic mucilages have been reported at several other Mediterranean sites, such as the Greek (Gotsis-Skretas, 1995; Nikolaidis et al., 2006) and Catalan coasts (Sampedro et al., 2007) where *Gonyaulax fragilis* was thought to be involved in their production, and the Sea of Marmara (Turkey) where

Cylindrotheca closterium, *Skeletonema costatum* and *Gonyaulax fragilis* were indicated as the most abundant species (Tüfekçi et al., 2010). In the Tyrrhenian Sea, extensive pelagic aggregates were observed in 1991, 2000 and 2012 (Figure 3- 5, (Calvo et al., 1991; Escalera et al., 2018; Innamorati et al., 1993)). Foam accumulated massively along the Catalan coast in March 2006 during a *Phaeocystis* sp. bloom, an event that was related to anomalous hydrographic winter conditions (Arin et al., 2014).

Massive mucilage events have also concerned the benthic environment. *Ostreopsis* cf. *ovata* during intense blooms forms a network-shaped mucilaginous biofilm that can harm benthic invertebrates (Schiaparelli et al., 2007). In the Tyrrhenian and Ligurian Seas (western MS), benthic mucilages have occurred since 1991 (Sartoni and Sonni, 1991), and have been attributed to the massive growth of several macro- and microalgae such as the filamentous brown alga *Acinetospora crinita* and the colonial pelagophytes *Nematochrysopsis marina* and *Chrysonephos lewisii* (Giuliani et al., 2005; Schiaparelli et al., 2007). The allochthonous pelagophyte *Chrysophaeum taylorii*, recorded in the western MS since 2005, in recent years was involved in the formation of dense layers of mucous covering macroalgae, gorgonians and the surrounding rocks (Caronni et al., 2015; Luglié et al., 2008).

3. Trends in the Mediterranean HABs

3.1. General trends

The MS has undergone profound changes over the last centuries. Human action has mainly been visible along the coasts of the basin, which have become increasingly populated and deeply modified by coastal and riverine engineering and deforestation which, along with cultural eutrophication, are all potential drivers of deep changes in phytoplankton communities (Garcés and Camp, 2012). Natural and/or man-induced meteorological and climatic variations superimpose to these changes often with an amplifying effect. The most striking characteristic of the MS HABs over the last 50 yrs, which approximately correspond to the time since when they have been studied more intensively, is the remarkable increase of the toxic species list, from a few taxa to the more than 80 of the present review (Figure 3- 2). Over the same period, the records of these species across the MS have also remarkably increased (Figure 3- 6). This trend is parallel to that of the increased list of toxic species and of their records worldwide, which is an obvious result of the intensification of the taxonomic and toxin studies on marine microalgae (Zingone et al., 2017). The increase of the records of actual HAB events from the less than 30 cases listed by Jacques and Sournia (1978-1979) and Honsell et al. (1995) to the several hundred cases of halted aquaculture operations, seawater discoloration and minor human health accidents presently recorded in HAEDAT is also impressive (Figure 3- 7). Damages to aquaculture caused by ASP and PSP toxins in mussels have been limited over the last 30 years while DSP cases have represented about 75% of the harmful events, with an increase between the decade 1987–1997 and the two following ones (Figure 3- 7). This trend should however be interpreted with caution because it has been paralleled by a remarkable growth of the coastal MS population (Section 1), much more intensive use of marine resources, and consequent raise of the level of attention to the integrity and safety of marine resources.

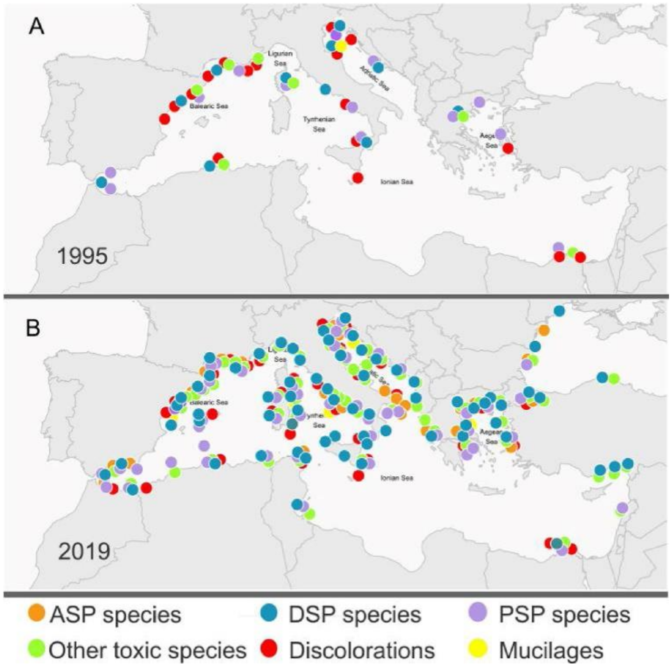

Figure 3- 6. Distribution of potentially toxic species, mucilages and discolorations in the Mediterranean Sea. A) Distribution of species known to be toxic and harmful events until 1995 as reported in Jacques and Sournia (1978-1979) and Honsell et al. (1995). B) Distribution of potentially toxic species (excluding *Ostreopsis* and CFP species) and harmful events updated to the present status of knowledge. The position of the circles in several cases has been slightly modified to reduce overlapping.

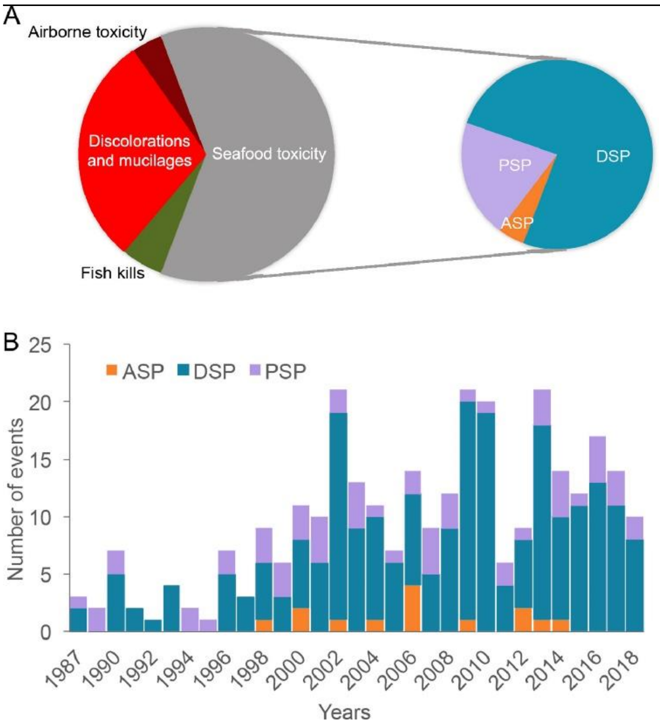

Figure 3- 7. Harmful events related to microalgae in the Mediterranean Sea (n = 501) based on records in the Harmful Algae Event Database HAEDAT (http://haedat.iode.org/). High density phytoplankton blooms with no impacts were not considered. A) Relative abundance of different types of nuisance with details of seafood toxicity. B) Interannual variations of ASP, DSP and PSP toxicity events.

In fact, toxic blooms as well as mucilage events and discolorations in the MS have generally shown an unpredictable interannual periodicity, like in the case of the conspicuous blooms of *Noctiluca scintillans* in the Adriatic Sea (Fonda Umani et al., 2004), Moroccan (Tahri-Joutei et al., 2003), Catalan (Lopez and Arte, 1971) and French coasts (M.-O. Soyer in Jacques and Sournia (1978-1979)). There are cases of decreases, e.g., the blooms of *Alexandrium pacificum* occurring on the Catalan coast from 1996 to 1998 (Vila et al., 2000) but rarely recorded afterwards (Sampedro i Roig, 2018). Blooms of *A. minutum* were recurrent in Egyptian waters but not recorded any longer after 1994 (Ismael and Halim, 2000), while their frequency doubled from 2000 to 2012 along the Catalan coast (Sampedro i Roig, 2018). Blooms of the ciliate *Mesodinium rubrum* hosting cryptophyte chloroplasts were not recorded in the MS (Jacques and Sournia, 1978-1979) until their occurrence in both the Adriatic (Sorokin et al., 1999) and Tyrrhenian Seas (Siano et al., 2006), and afterwards have only been observed in 2017 in the North Aegean Sea (Genitsaris et al., 2019).

In the case of *Ostreopsis* cf. *ovata*, rather than an increase the phenomenon in the MS has shown a sudden upsurge around the 2000, followed by an expansion of the known range for the species in the next years and a relative stability in the following decade. Indeed *Ostreopsis* cf. *ovata* provides the most evident case of range expansion and increased impact over time in the MS. Although benthic microalgae have received scarce attention until the late 20th century, it is unlikely that the species might have been abundant but undetected before. The apparent sudden range expansion and impact of *Ostreopsis* cf. *ovata* is in line with an increasing trend of species of the same genus in New Zealand and some other temperate areas around the world (Parsons et al., 2012). On the other hand, no clear increase of the impact or of species abundance has been reported since the 2000 outburst, while the above-mentioned range expansion has coincided with a dramatic increase in monitoring programs and research projects focused on benthic microalgae. Initially, the sudden relevance of the phenomenon was associated with an increase of temperature in the MS, based on the belief that all *Ostreopsis* species were of tropical origin. In fact, *Ostreopsis* cf. *ovata* and its close relatives are widely distributed in temperate areas, also matching the apparent preference of the species for moderately high rather than very high temperature (Mangialajo et al., 2011; Scalco et al., 2012). Overall, the trend observed for this species in the MS, with an outburst followed by a stabilizing trend, recalls that of an invasive species rather than that of a species favored by a temperature increase.

3.2. HAB trends in the Adriatic Sea, a case study

The Adriatic Sea (AS) represents a unique system for its semi-enclosed morphology, shallow depth and oligotrophic nature in most parts but with eutrophic characteristics along the north-western coasts driven by inputs from the Po River and other rivers (Cozzi and Giani, 2011; Mozetič et al., 2010). The AS is considered one of the hotspots of MS HABs (Garcés and Camp, 2012), in terms of both occurrence and impacts. However, compared to the great variety of potentially toxic species (Mozetič et al., 2019), toxicity cases are limited, and the most common toxins found above the regulatory limits in the Adriatic shellfish to date are DSP toxins (okadaic acid group) and other lipophilic toxins (yessotoxins and pectenotoxins).

Because of the early development of sea-related activities, there is a wealth of information from the area dating back to the last century, which allows some insights on possible HAB trends. Phytoplankton in certain areas of the AS (e.g., Gulf of Trieste, Gulf of Venice, Senigallia-Susak transect, Kaštela Bay) have been extensively studied for decades (Bernardi Aubry et al., 2012; Cerino et al., 2019; Marić et al., 2012; Mozetič et al., 2012; Ninčević Gladan et al., 2010; Totti et al., 2019b), highlighting a number of changes, such as trends or regime shifts in main phytoplankton groups (Mozetič et al., 2010; Totti et al., 2019b) and in bloom forming species (Cabrini et al., 2012). However, no trends specifically related to toxic species is evident from these long-term studies, neither in terms of increased frequency nor of abundance. In fact, most studies on HAB species are snapshots of isolated toxic episodes (Pistocchi et al. (2012), and references therein). Similar conclusions can be drawn also from toxicity events: aquaculture operations have been halted frequently over the last 20 years (Section 2.2.1), but without any significant trend for DSP events.

Nevertheless, some changes in phytoplankton community structure of the AS have involved a number of HAB species, such as *Pseudo-nitzschia multistriata*, an allochthonous species (Section 2.1) that became a regular component of the autumn phytoplankton communities of the NW AS (Totti et al., 2019b). In the Gulf of Trieste, previously rare *Dinophysis tripos* have become a regular member of the autumn phytoplankton assemblages since 2010, along with higher temperatures recorded in this decade (Francé et al., 2018), whereas further south *D. sacculus* has replaced *D. caudata* as one of the indicator species of spring phytoplankton communities (Totti et al., 2019b).

HB-HABs caused by dinoflagellates, occurring in summer and often associated with water discoloration and bottom anoxia, were a major problem in the AS until the end of the 1980s (see Section 2.3.1). At the time, because of the heavy impact on the local economy, the Italian government adopted countermeasures to reduce P content in detergents and improve the urban wastewater treatment plants, leading to a strong reduction of P load in coastal waters. Since the end of the 1980s, summer dinoflagellate blooms became a rarer phenomenon, their decline coinciding with the years of large mucilaginous macroaggregate appearance.

Mucilages in the AS (see Section 2.3.2) were known since the beginning of 1700, when they were named ‘mare sporco’. In more recent years, massive episodes have occurred in the years 1988 to 1991 and 1997 to 2004, typically in summer (Giani et al., 2005), while a spatial and temporal reduction occurred in subsequent years. An anomalous occurrence in autumn-winter was reported in 2006–2007, probably in relation to a water temperature increase (Danovaro et al., 2009). The mucilage appearance, and the concurrent disappearance of summer water discolorations have both been associated with the decrease of inorganic and organic P (Degobbis et al., 2005), but also to hydrographic changes related to large-scale climatic changes around the end of the ‘80 s, which could have driven a regime-shift affecting not only the AS but also other European Seas (Conversi et al., 2010).

In the last decade (2008–2018), HB-HABs of both diatoms and dinoflagellates occurred without a regular temporal pattern, reflecting the meteorological events that nowadays tend to be more intense and unhampered by a regular seasonal rhythm (Totti et al., 2019a; Totti et al., 2019b). Blooms of *Fibrocapsa japonica* that were common at the end of the 1990s seem to be rarer since 2012 (Regione Emilia-Romagna, 1982-2018), and mucilage events only occurred shortly in 2014 and in 2018 (Regione Emilia-Romagna, 1982-2018).

As a whole, HABs in the AS show unpredictable time variability that is partly related to the irregularity and intensity of meteorological events in the last decades. Prolonged periods of drought (Cozzi et al., 2019) with oligotrophic conditions (Mozetič et al., 2010) alternate with nutrient pulses from continental water runoff that can drive the occurrence of anomalous intense blooms at any time of the year (Totti et al., 2019b).

4. Conclusions

A deep knowledge on the spatial and temporal distribution of harmful species and the blooms that they produce is an indispensable goal towards a safe use of marine resources and an informed management and planning of the coastal zone. In the MS this goal is even more crucial considering the importance of the economy deriving from the use of the sea for tourism and recreational use, fishery and aquaculture. The information about HABs has grown remarkably over the last 50 years since the first review (Jacques and Sournia, 1978-1979) all over the MS areas. However, the marked west-east and north-south gradients in the knowledge of HABs and HAB species distribution persist, with long traits of coast with scarce or no information available.

Overall, the MS hosts a high number of potentially toxic species, many of which have a wide distribution across its coastal waters. Yet the cases of intoxication are extremely rare, while the impact on aquaculture appears to be limited to a few hot spots in the northern Adriatic, Spain and France coasts. A variety of toxins have actually been detected in several instances in microalgae strains from the MS, while seafood toxicity, when detected, has commonly remained below the safety limits. The typical oligotrophic offshore Mediterranean waters that influence most coastal areas and the enhanced alongshore circulation in many places may play a role in keeping toxic algae at levels rarely exceeding critical density thresholds, thus preventing their excessive accumulation in seafood. On the other hand, quite effective monitoring operations have accompanied the development of aquaculture over the last decades, thus reducing the possibility of accidents to a minimum level.

In terms of microalgal toxins, the only major concern seems to reside in the large amount of palytoxin-like substances that every summer accumulate along the rocky Mediterranean shores because of *Ostreopsis* blooms. Although sea urchins and wild mussels inhabiting those environments at time accumulate those toxins to considerable levels, no cases of seafood intoxication have occurred so far. Contaminated herbivorous fishes represent a problem in areas where they ingest macroalgal substrates colonized by toxic microalgae, i.e. in the ciguatera areas, but species capable of this transfer link may be missing in the MS trophic webs, or toxins are neutralized in the transfer. Nonetheless, the guard level must be kept high because sudden changes might occur, e.g., due to penetration of benthic herbivorous fish in the MS and consequent novelties in the local food webs.

Overall, the present overview demonstrates a relatively low risk deriving from toxic blooms and a higher risk from high biomass blooms affecting the aesthetic qualities of coastal areas devoted to tourism in the MS. No clear trends in occurrence nor expansions emerge for either toxic or HB-HABs. While EU regulation and national initiatives have promoted actions addressing seawater quality and aiming at a good environmental status (GES), human densities along the coasts is predicted to keep on increasing in the next decades. Therefore, a larger use of marine resources in the future, in the MS like in other coastal areas of the world, will probably lead to an increased impact of the risks posed by HABs even in absence of any trends in their abundance and frequency (Zingone and Wyatt, 2005). In addition, predicted changes in climate and consequent modifications of hydrographical features may drive local variations in microbial populations both in the plankton and in the benthos. Continued monitoring and further studies on HAB patterns and trends are therefore mandatory goals to be able to predict their evolution and protect human health and wellbeing in the MS.

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Chapter 4. New pathologies.

The contents of this chapter were published in the journal Frontiers in Marine Science.

Title: The endoparasite *Perkinsus olseni* affecting the Mediterranean mussels (*Mytilus galloprovincialis*) in the Italian and Spanish waters: A new possible threat for mussel aquaculture and wild animal population.

Authors: Francesca Carella, Margarita Fernandez Tejedor, Grazia Villari, Karl Blyth Andree and Gionata De Vico.

Reference: Carella F, Fernandez Tejedor M, Villari G, Andree KB and De Vico G (2023) The endoparasite Perkinsus olseni affecting the Mediterranean mussels (Mytilus galloprovincialis) in the Italian and Spanish waters: A new possible threat for mussel aquaculture and wild animal population. Front. Mar. Sci. 10:1116837. doi: 10.3389/fmars.2023.1116837

Abstract

Dinoflagellates belonging to the *Perkinsus* genus are OIE (World Organization for animal Health)-listed pathogens extremely virulent for clams and oysters in many marine ecosystems throughout the world. During the monitoring activities of the Mediterranean mussel (*Mytilus galloprovincialis*) in Campania region (Italy), the presence of typical trophozoites of *Perkinsus* sp. was observed in mussels from farms and natural banks. Simultaneously, following mussel mortality in the Spanish waters of Catalonia, histopathological studies revealed the presence of the same parasite. Although perkinsosis is an endemic disease in clams in Italy (with prevalence from 40 to 80 %), there are no reports to date of its presence in Mediterranean mussels and of the effect on this species. For this study, histopathology, Ray's Fluid Thioglycollate Medium (RFTM), and molecular diagnostics with conventional Polimerase Chain Reaction (PCR) and qPCR were performed. In samples from Italy, histopathology in the mussel from one farm revealed a prevalence of 26 % in February 2019, 40 % in February 2020, 16 % in November 2020, and 23 % in April 2021. In a natural bank, *Perkinsus* was also detected in May 2020 but in lower prevalence. In Spain, in July 2020, the presence of the parasite was 20 % in one site and 10 % in a second site and related to animal mortality. In both areas, *Perkinsus* sp. elicited multiple inflammatory capsules of different size or infiltrates at the level of the digestive gland and gonad. Molecular diagnostics of the Internal Transcriber Spacer (ITS) region of the rDNA (ITS1, 5.8S, and ITS2) showed a 97 % similarity of *P. olseni* from Italy with samples from New Zealand, Australia, and Uruguay and in bivalves such as *Pitar rostrata*, *Astrovenus* sp., and *Haliotis* sp., whereas in Spain the identity was 99 % samples from South Korean venerids such as *Anadara granosa*. Phylogenetic analysis group together *P. olseni* from Italian and Spanish mussels but place them distant from other *P. olseni* described in the clams from Europe (Italy, France, and Spain). Direct impact of transboundary animal diseases in aquaculture constitutes a serious consequence for export living animals and their products, as well for international trade. This compromises food security, also causing a high socioeconomic impact on aquaculture exporting nations. *P. olseni* is a generalist pathogen able to infect different bivalve species, possibly passing from clams to oysters and mussels. Recognized international organizations should take this into account in the view of possible cross-infection. Other studies are needed to define pathogen virulence in this species.

1. Introduction

In the past years, infectious diseases are emerging significantly in marine and freshwater environments. The elements involved in this emergence are different. Cultivated animals are involved in the global trading, facilitating the introduction of serious infectious diseases, called transboundary diseases (TD). Several transboundary aquatic animal diseases (TAADS) have swept regions over the past 30 years causing massive economic and social losses, responsible for the introduction, establishment, and spread of pathogens into new geographic areas. Nowadays, there are several international codes of practice and guidelines to reduce the risk of introducing pathogens. WOHA (World Organization of Animal Health) has developed recommendations and protocols in the International Aquatic Animal Health Code, which deals with the health surveillance of aquatic animals (OIE, 2021).

Emerging disease in mussels has been reported repeatedly in the past years, as in other bivalve species. Recently, the potentially zoonotic bacteria *Nocardia crassotreae* were reported in the Mediterranean mussel *M. galloprovincialis* (Carella et al., 2013a; De Vico and Carella, 2019), the OIE listed parasite *Marteilia refringens* have been observed in the area (Carella et al., 2010), and many other emerging disease conditions have been also reported in other bivalve species in the same area (Carella et al., 2013a; Carella et al., 2013b).

Perkinsosis is an important disease that has been reported worldwide in bivalves and gastropods. *Perkinsus* pathogens can infect a wide range of hosts and possibly are responsible for mortality events for their extensive invasive ability and virulence. Nowadays, seven species within the genus *Perkinsus* have been reported including *P. marinus*, *P. olseni*, *P. qugwadi*, *P. chesapeaki*, *P. mediterraneus*, *P. honshuensis*, and *P. beihaiensis* (Ramilo et al., 2015) with only *P. olseni* and *P. marinus* listed notifiable parasites listed by OIE (OIE, 2021).

Pathogens can display a highly flexible ranges of hosts, called multi-host or generalist pathogens, or can infect only one or a few related species and called specialist pathogens. *P. olseni* has been reported in 30 mollusc species, bivalves, and gastropods over a wide range of geographical locations (Itoiz et al., 2022). It is generally associated with mass mortality of clams such as Manila clams *Ruditapes philippinarum* in Europe, the venerid clam *R. philippinarum* in Asia, the cockle *Austrovenus stutchburyi* in New Zealand (Dungan et al., 2007), and in the abalone *Haliotis* spp in Australia. Recently, reports of perkinsosis in mussels have been increasing; Itoh et al. (2019) reported *P. beihaiensis* infection in the invasive *M. galloprovincialis* in Japan, whereas Vazquez et al. (2022) reported *P. olseni* in *M. chilensis* in Argentina.

In this study, we report, for the first time, the presence of the parasite *Perkinsus* sp. in the Mediterranean mussel *M. galloprovincialis* in Europe. First detection was in mussels from Italy, in Campania region, in mussel farms from 2019 to 2021 and later in natural beds in 2020. During the study, we also observed the presence of *Perkinsus* sp. like cells in mussel samples from Catalonia (Spain) following a mussel episode of mortality. Within the past few years, more data on the genetic variation within some of the *Perkinsus* species have become available, and many ITS (internal transcribed spaces) regions now described have that allowed to assess intraspecific variation and to compare the dissimilarity of sequence with the differences observed among the *Perkinsus* species.

During the surveys, we conducted phylogenetic analyses to estimate the relationships with the group of *Perkinsus* spp. in mussels from Italy and Spain and haplotype characterization along with animal histopathology to define host response and possible *Perkinsus* pathogenicity.

2. Materials and methods

2.1. Sampling of Mediterranean mussels in Italy and Spain

During 2018–2021, a field survey targeting infectious agents of the Mediterranean mussel Mytilus galloprovincialis was conducted in which mussels were manually collected in one mussel farm and one natural bank on the north coast of the Campania region (Italy).

In Italy, collections were made in a mussel farm in Campania Region (Naples Bay) in February 2019 (N = 20), February 2020 (N = 20), November 2020 (N = 30), and April 2021 (N = 30). A sampling was performed in a close natural bank in May 2020 (N = 30). Alfacs Bay, located in the south of the Ebro delta (Western Mediterranean), is a shellfish growing area where mussels and oysters are grown in ropes hanging from rafts. Sampling in Alfacs Bay was conducted on 21 July 2020, 2 weeks after the beginning of the mortality event on 6 July 2020 as reported by the mussel farmers. Sampling in Alfacs Bay was conducted in two sites, A (40°37’15.28”N; 0°39’14.58”E) and B (40°37’1.98”N; 0°37’51.66”E) (Figure 4- 1). The mussels were transported to the laboratory alive in isothermal boxes. Prior to processing, the animals were measured for animal shell length, total weight, and meat weight (MW) according to Galtsoff (1964).

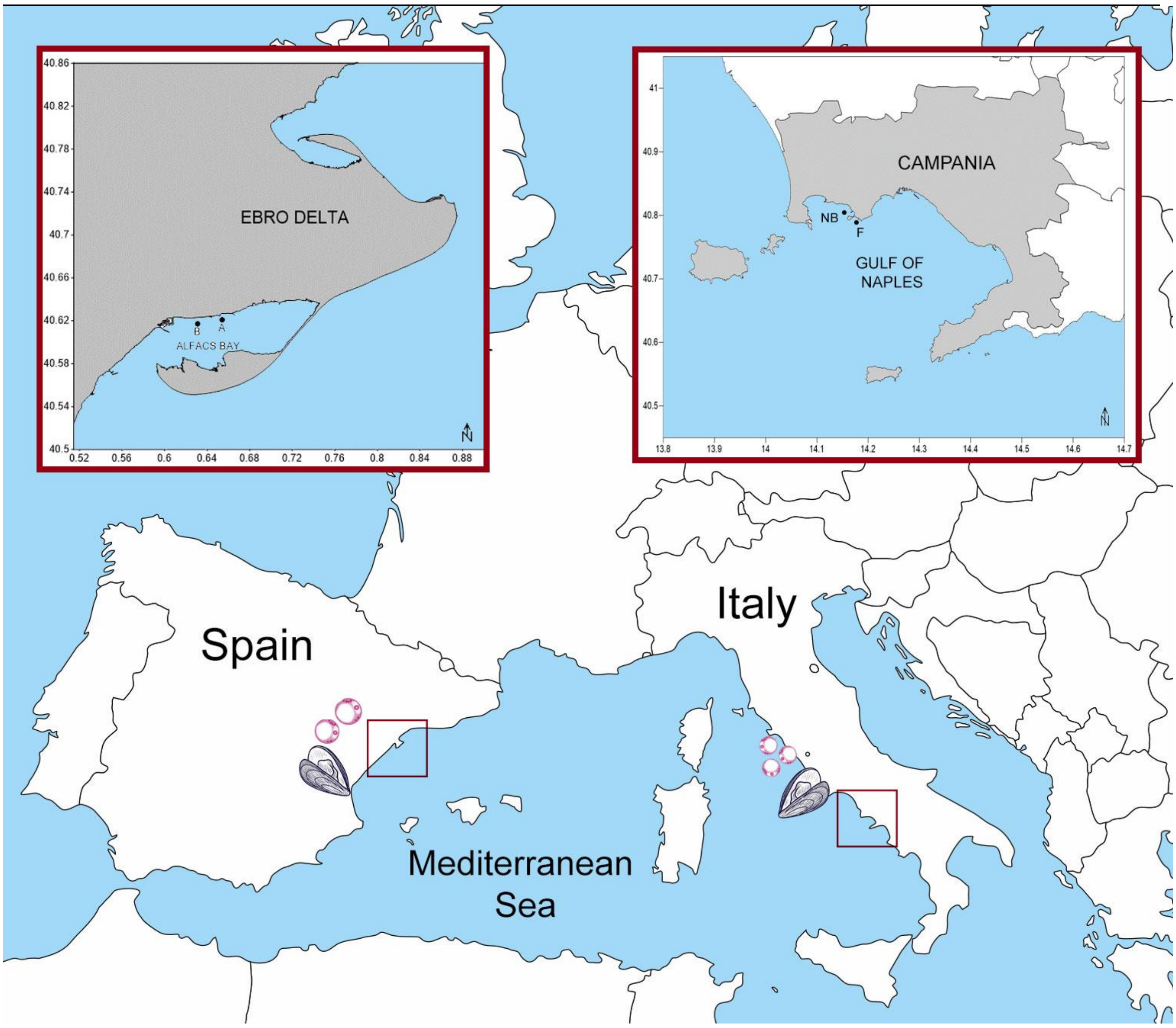


Figure 4- 1. Locations of sampling areas in Italy and Spain: Bay of Naples and Bay of Alfacs.

2.2. Light microscopy

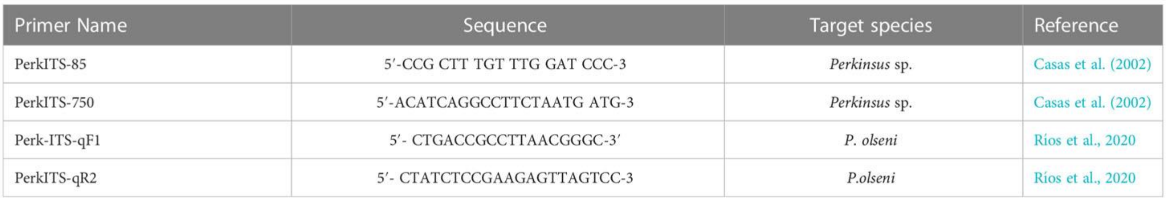
For animal histopathology, from each animal, a transverse section including digestive tissue, gill, foot, and gonad was obtained and fixed in Davidson's solution for 72–96h. Fixed tissues were embedded in paraffin blocks and sectioned at 3 µm with a rotary microtome (Bioptica, Italy). Tissue sections were deparaffinised, stained with Carazzi haematoxylin and eosin and a special stain such as Mallory's trichrome (Mazzi, 1977). Digital images and measurements were obtained using an integrated Axioscope A1 (Zeiss, Germany) and camera Axiocam 208.

2.3. PCR and qPCR for *Perkinsus* species identification and presence evaluation

From each specimen, 25–30 mg of gonad, digestive tissue and gills, preserved in TE buffer at −20°C, was taken for total genomic DNA extraction using QIAamp DNA Mini Kit (QIAGEN, Germany), according to the manufacturer's instructions (tissue protocol). The DNA quality and quantity were measured with the use of a Nanodrop spectrophotometer (Thermo Fisher Scientific) and stored at

−80 °C for long-term preservation. Primers used in the study are listed in Table 4- 1. More in detail, PCR assays using the generic primers PerkITS750/PerkITS85 (Casas et al., 2002) were carried out first to detect *Perkinsus* spp. in all sampled mussels. PCR reactions were carried out in 50 µl of final volume using the Mastermix GoTaq polymerase (Promega) following the instructions of the manufacturer. A positive control provided by IRTA institute constituted by a clam infected *P. olseni* was included in each reaction along with a negative control (master mix with no DNA). Amplification parameters were performed as follows: An initial denaturation of 4 min at 94°C followed by 35 cycle amplifications (1 min at 94 °C, 1 min at 53 °C, and 3 min at 68 °C) and a final extension of 5 min at 68 °C. The resulting PCR products were purified and sent to an external sequencing facility (Eurofins Genomics, Germany).

Table 4- 1. List of the primers used to detect *Perkinsus* spp. with PCR and qPCR in this study.



Primer Name	Sequence	Target species	Reference
PerkITS-85	5′-CCG CTT TGT TTG GAT CCC-3	*Perkinsus* sp.	Casas et al. (2002)
PerkITS-750	5′-ACATCAGGCCTTCTAATG ATG-3	*Perkinsus* sp.	Casas et al. (2002)
Perk-ITS-qF1	5′- CTGACCGCCTTAACGGGC-3′	*P. olseni*	Ríos et al., 2020
PerkITS-qR2	5′- CTATCTCCGAAGAGTTAGTCC-3	*P.olseni*	Ríos et al., 2020

To better define pathogen presence in the Italian samples, a more sensitive procedure of real-time quantitative PCR (qPCR) was also performed using primers Perk-ITS-qF1/Perk-ITS-qR2 (Rios et al., 2020) that amplifies the internal transcribed spacer region (ITS-1 and ITS-2) of the gene complex that codes for ribosomal RNAs in *P. olseni*. Wells were filled to a final volume of 10 µl, using 1 µl of DNA, 5 µl of Taq Universal SYBR green mix (Applied Biosystem), 0.5 µl of each primer (10 µM), and 3 µl of distilled water. Amplification was performed under the following conditions: denaturation for 10 min at 95 °C, amplification by 40 cycles of 15 s at 95 °C and 60 s at 60 °C, melting curve evaluation 1 min at 95 °C, and increase of 0.5 °C each 30 s starting in 60 °C, end at 95 °C for 15 s. All reactions were performed using two technical replicates.

2.4. Phylogeny and haplotype analysis of *Perkinsus* based on the ribosomal ITS region

The resulting ITS chromatograms (648 bp) were analyzed using BioEdit software (v. 7.2). All generated sequences were searched for identity using BLAST (Basic Local Alignment Search Tool) through web servers of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The sequences were also aligned with the available sequences for *Perkinsus* spp. found in the GenBank database using the MUSCLE algorithm. Maximum likelihood (ML) analysis was conducted using MEGA version X software (Kumar et al., 2004) with 1,000 replicates for calculating bootstrap values.

For the haplotype network analysis, ITS1 sequences of *Perkinsus* obtained from Campania samples (n = 2) and Catalonia (n = 2) and 163 ITS1 sequences of *P. olseni* with geographic information deposited in GenBank were used. Using MEGA X software, sequence data were aligned by CLUSTAL W (Thompson et al., 1994) at default settings. The haplotype network among 163 ITS1 sequences of *P. olseni* was constructed with the TCS network method (Clement et al., 2000) using PopART (Leigh and Bryant, 2015).

2.5. Ray's fluid thioglycollate medium RFTM assay

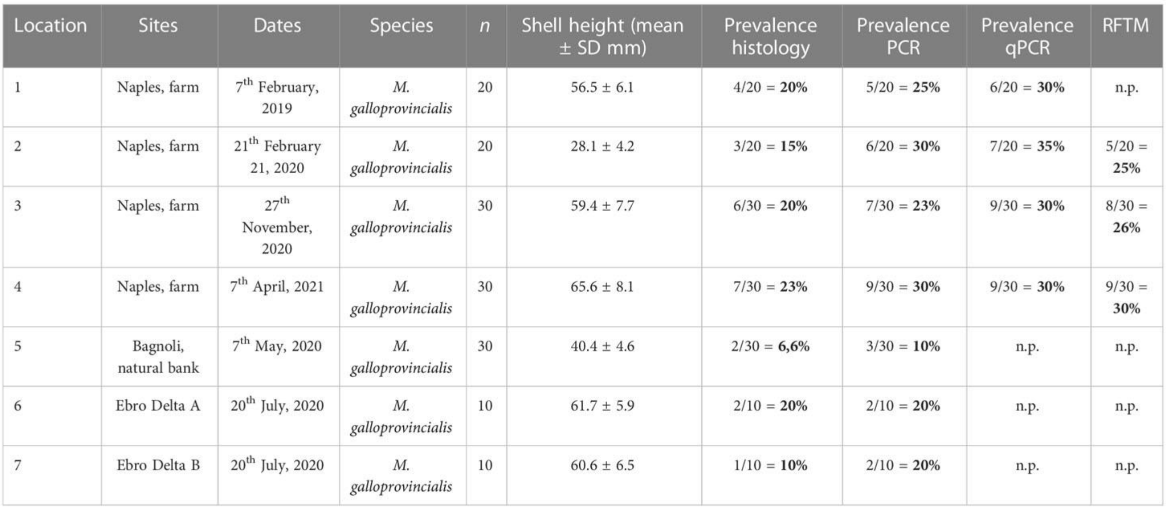
RFTM has been considered the best assay for *Perkinsus* diagnosis (Ray, 1963). In the mussel farm from Italy, starting from the samples of 2020, aseptically excised small pieces (3–5 mm) of digestive gland, gill, mantle, and muscle were in RFTM supplemented with Chloramphenicol 2,5% w/v and Nystatin (4000 U ml−1) in the dark, at for 6 days at 26°C. After incubations, the tissues were placed on a glass slide, covered by a drop of Lugol's iodine solution, cover-slipped and examined under a light microscope (Zeiss Axioscope 5) at different magnification (4×, 10×, and 20× objectives). The sample resulted positive when blue–black hypnospores were observed.

3. Results

3.1. Animal histopathology

Perkinsosis prevalence in mussels from Italy was variable over the years and seasons by light microscopy (Table 4- 2). The highest prevalence was detected in April 2021 with the 23 % of the affected individuals.

Table 4- 2. Survey results of *Perkinsus* infection in the blue mussel, *M. galloprovincialis* collected from Campania region and Ebro Delta. n.p.: analysis not performed.



Location	Sites	Dates	Species	*n*	Shell height (mean ± SD mm)	Prevalence histology	Prevalence PCR	Prevalence qPCR	RFTM
1	Naples, farm	7th February, 2019	*M. galloprovincialis*	20	56.5 ± 6.1	4/20 = **20%**	5/20 = **25%**	6/20 = **30%**	n.p.
2	Naples, farm	21th February 21, 2020	*M. galloprovincialis*	20	28.1 ± 4.2	3/20 = **15%**	6/20 = **30%**	7/20 = **35%**	5/20 = **25%**
3	Naples, farm	27th November, 2020	*M. galloprovincialis*	30	59.4 ± 7.7	6/30 = **20%**	7/30 = **23%**	9/30 = **30%**	8/30 = **26%**
4	Naples, farm	7th April, 2021	*M. galloprovincialis*	30	65.6 ± 8.1	7/30 = **23%**	9/30 = **30%**	9/30 = **30%**	9/30 = **30%**
5	Bagnoli, natural bank	7th May, 2020	*M. galloprovincialis*	30	40.4 ± 4.6	2/30 = **6,6%**	3/30 = **10%**	n.p.	n.p.
6	Ebro Delta A	20th July, 2020	*M. galloprovincialis*	10	61.7 ± 5.9	2/10 = **20%**	2/10 = **20%**	n.p.	n.p.
7	Ebro Delta B	20th July, 2020	*M. galloprovincialis*	10	60.6 ± 6.5	1/10 = **10%**	2/10 = **20%**	n.p.	n.p.

In Italy, typical features of *Perkinsus*-like cells with a trophozoite characterized by a large vacuole and eccentric nucleus were generally visible, accompanied by the typical production of many inflammatory capsules of different dimensions and with an infection intensity from moderate to high (Figure 4- 2). Chronic lesions, underlined by the involvement of fibroblast, were observed in samples from April 2021. The parasite developed in the connective tissue in the digestive gland, between the tubules, in the connective stroma in the gonad, and in few cases in the muscle of the foot. Within the capsule were visible haemocytes phagocytising at different phases of development were visible, from multinucleate schizonts from four to eight cells. Apoptotic haemocytes were also visible along with the production of yellowish granules (Figure 4- 2). In Spain, sites A and B showed animal mortality, 69 % in site A and 17% in the site B. The infection was visible through histopathology in two individuals (2/10) in the site A and in one individual in site B. The inflammatory lesion was intense and infiltrative, spreading in all the digestive tissue, with total disappearance of tissue architecture. Moreover, differently from animals in Italy, trophozoites of *P. olseni* were also observed in the gill, a typical *Perkinsus* spp. tropism, and in the haemocyte vessels (Figure 4- 2). Clusters of trophozoites were encapsulated in well-circumscribed walls forming a cyst-like structure.

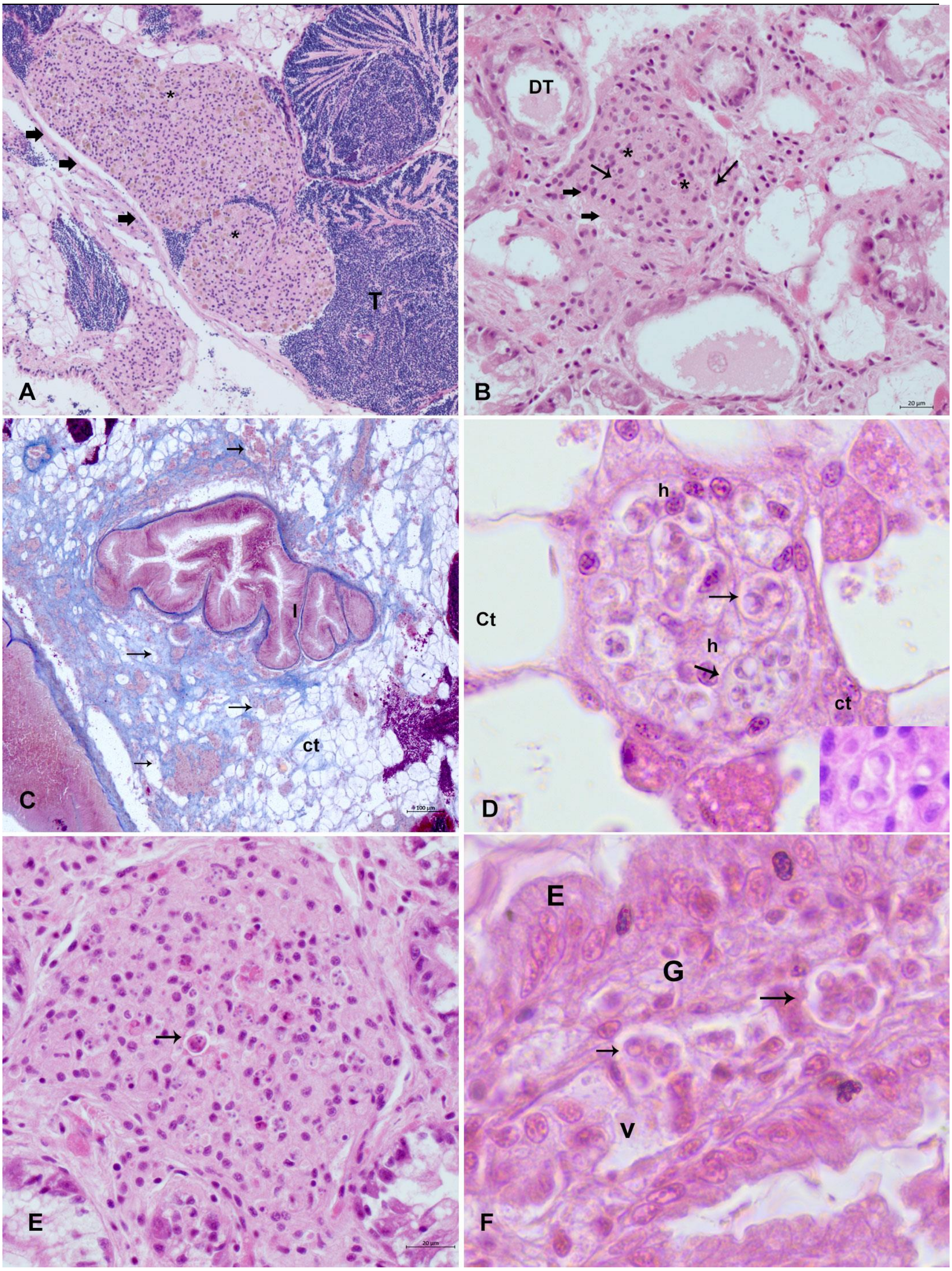


Figure 4- 2. Histopathology of mussels affected by Perkinsosis in mussels (*M. galloprovincialis*) in Italy and Spain. (A, B) typical feature of the inflammatory lesion (big arrows) with haemocytes (*) related to *Perkinsus* (small arrows): haemocytes nodulation in gonadal follicle (A) and the interstitial space of digestive tubules (B) big arrows. (C) detail of the reactive connective tissue, underlined by Mallory Trichrome in light blue with inflammatory capsules (arrows); (D) detail of a capsule in the connective tissue space with haemocyte (h) phagocyting trophozoite of *Perkinsus* (arrow); (E) inflammatory

capsule displaying apoptotic haemocytes (arrow) with visible trophozoite. (F) *Perkinsus* (arrows) in the gill (G) haemal vessel in samples from Spain; E, epithelium.

3.2. Disease diagnosis with RFTM, PCR, and qPCR

After 6 days, RFTM-cultivated infected mussel stained with Lugol's iodine exhibited dark blue/black hypnospores in the digestive gland and mantle tissue and rarely in the gill (Figure 4- 3). In mantle, muscle, and digestive gland, hypnospores were observed as clusters when the intensity was low or completely dispersed. Comparison with PCR, qPCR, and cultivation methods in the two areas showed variable prevalence. In Italy, qualitative PCR reported values from 10 to 30% while detection was higher using qPCR values between 30 and 35 %. RFTM detection was comparable with the qPCR although always more efficient than PCR from Casas et al. (2002). In Spain, PCR found positive samples in 13.30 % of the animals in the two areas.

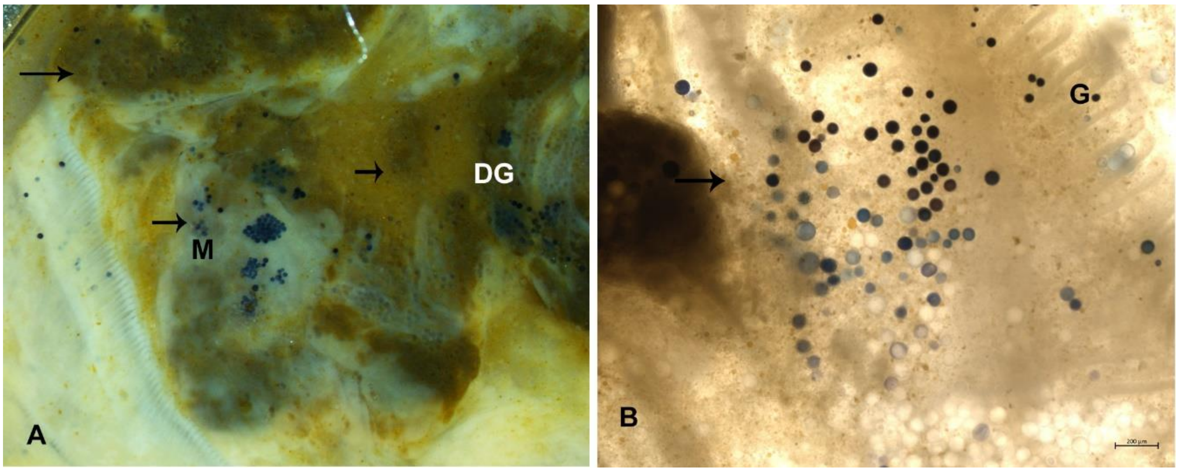


Figure 4- 3. Ray's fluid thioglycolate medium (RFTM) assay. (A) *Perkinsus* hypnospores in very heavy infection in *M. galloprovincialis* in Campania in digestive tissue (DG), muscle (M) and mantle (A) and connective tissue close to the gills (G) (B).

3.3. Molecular analysis: *Perkinsus* phylogeny and haplotype

The infected animals resulted in positive detection for the genus-specific primers for *Perkinsus* with a 648 bp amplicon. The species identity was checked by sequencing followed by BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In samples from Italy, BLAST analysis showed a 96% similarity of *P. olseni* with samples from New Zealand, Australia, and Uruguay and in bivalves such as *Pitar rostrata*, *Astrovenus* sp., and *Haliotis* sp., whereas in Spain, the identity was 99 % with samples from South Korea such as *Anadara granosa* (GenBank Accession number: OP961719-OP961722).

The phylogenetic tree resulting from Maximum likelihood is shown in Figure 4- 4. All *Perkinsus* spp. sequences from this study groups together *P. olseni* from Italian and Spanish mussels but places them more distant from other *P. olseni* described in the clams from Europe such as clams from Italy, France, and Spain. In Spain, in particular, they are distant from *Perkinsus* sp. such as those from Galicia and from sequences previously described from clams from the Ebro Delta. The genetic distance between these isolates and other isolates of *P. olseni* from different geographic locations ranged from 0.20 to 0.31. The pairwise genetic distance between the *P. olseni* isolates from Campania with those of Spain varied from 0.31 to 0.27, respectively (Table 4S- 1).

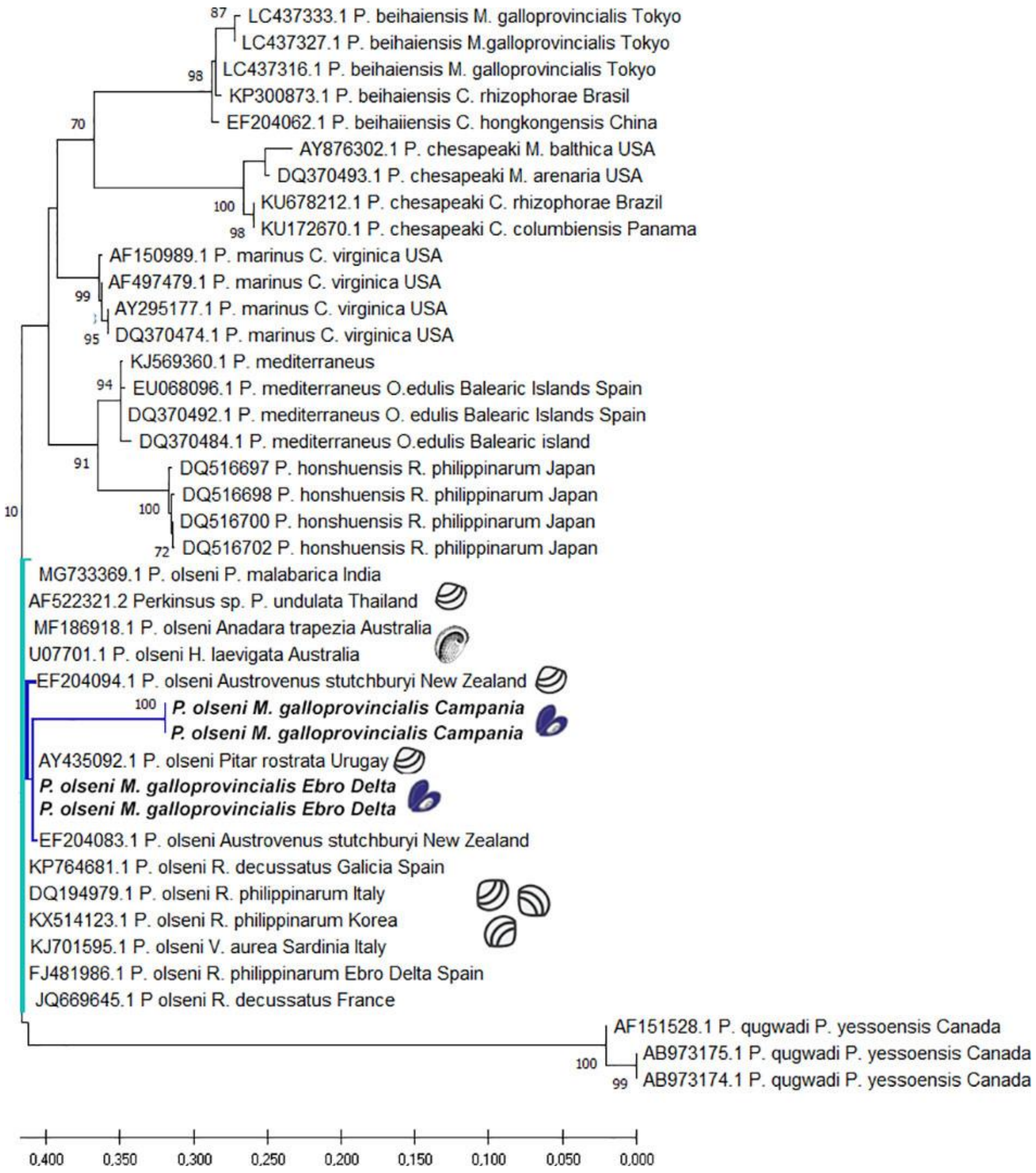


Figure 4- 4. Evolutionary analysis by Maximum Likelihood method of *Perkinsus* spp. ITS sequences. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 41 nucleotide sequences. There was a total of 794 positions in the final dataset.

We found 20 different ITS haplotypes among 163 ITS sequences (Figure 4- 5). One of the biggest haplogroups was composed of haplotypes from Asian countries (Korea, Thailand, and Japan), Europe (Spain, Italy, and France), and few sequences from Brazil. The other haplotypes were divided into the two major haplogroups, which were separated by at least three nucleotide differences. *Perkinsus* sp. in mussels from Aflacs Bay was grouped in another haplogroup containing sequences of bivalves from Europe, New Zealand, Japan, and Korea. The sequences from New Zealand belonged to the New Zealand cockle *Austrovenus stutchburyi*. *P. olseni* in Naples Bay belonged to an isolated haplogroup close to the Alfacs Bay haplogroup.

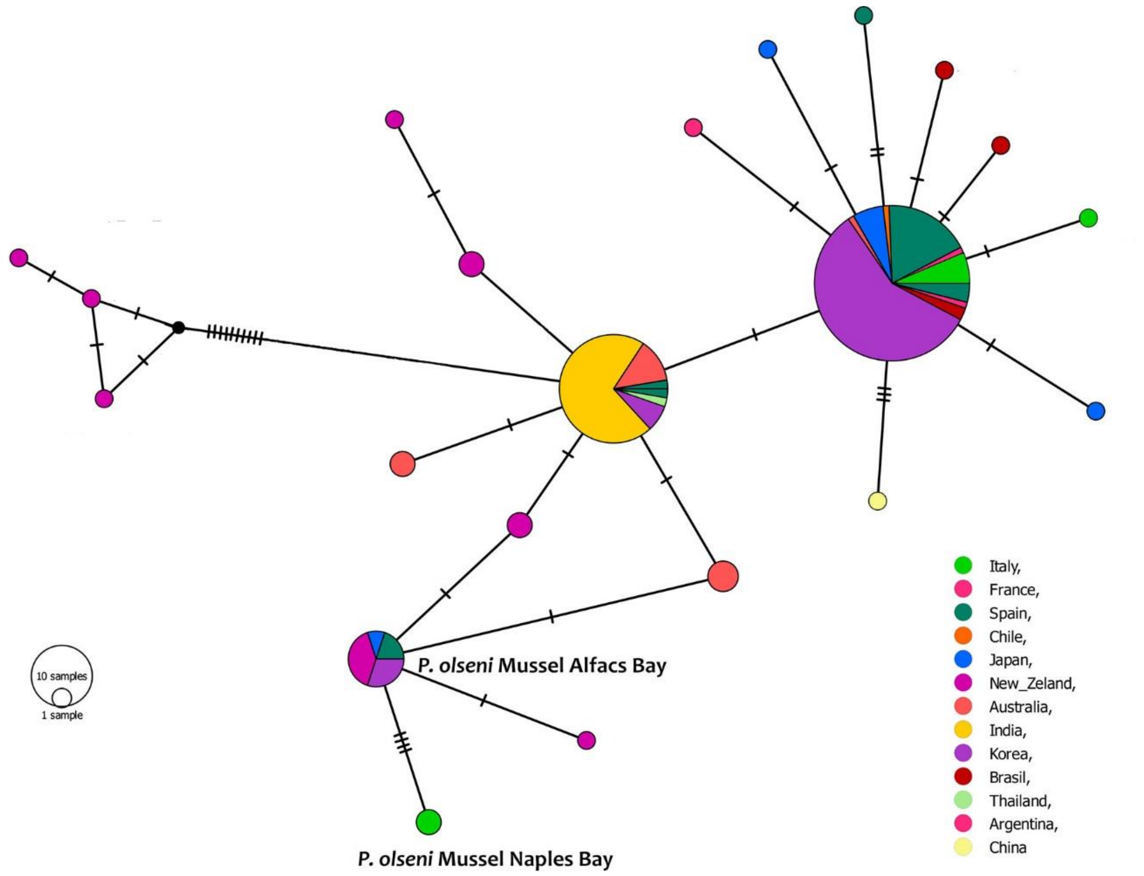


Figure 4- 5. TCS network of 163 ITS1 haplotypes of *Perkinsus olseni* estimated by PopART. Each line between haplotypes indicates a single nucleotide substitution. The size of each circle is proportional to the absolute haplotype frequency, small black circles represent missing haplotypes, and color shows localities where each haplotype was observed.

4. Discussion

This is the first report of *P. olseni* in *Mytilus galloprovincialis* in the Mediterranean Sea. Perkinsosis due to *Perkinsus olseni* in an OIE-listed disease impacts on the health and fitness of populations of many bivalve species (Villalba et al., 2004). Previous works reported the presence of *Perkinsus* species in other mytilidae group, in Japan and Argentina, also describing strong inflammatory lesions connected to the pathogen presence. *P. marinus* and *P. olseni* are the most devastating species and associated with massive mortalities and economic loss. *P. olseni* typically parasitizes Manila clams in Europe and Asia, the carpet shell *R. decussatus* in Europe as well as a the gastropod *Haliotis ruber* in the South of Australia (Lester and Davis, 1981).

Recent advancement of the in vitro culture of *Perkinsus* spp. improved the understanding of the biology of the parasite, its pathogenicity and virulence and the impact on the host like clams and abalone (La Peyre, 1993; Li et al., 2019; Soudant et al., 2013). On the other side, literature report that mussels could be less affected by infectious agents compared with other bivalves' species (Auguste et al., 2020; Moreira et al., 2020). Because of the scarce knowledge on mussel–*Perkinsus* relationship, it is critical to define the potential negative effect on this new host and consider pathogen prevalence, intensity, and aspects of the inflammatory response. Perkinsosis is a parasitic disease that develops essentially on an inflammatory basis. In clams, the parasite destroys the epithelia and damages the basal membranes of digestive tissues and gills. It is distributed in different tissues and organs, causing an intense inflammation characterized by the formation of infiltrates, nodules, and capsules, to surround and destroy the pathogen, incorporated in an abundant visible PAS-positive substance, a lectin, secreted by the haemocytes (Kim et al., 2006; Montes et al., 1995, 1996; Soudant et al., 2013). In mussels from this study, *P. olseni* showed different tropisms, rarely present in the epithelia, a feature observed in other bivalve species such as the blood cockle *Anadara kagoshimensis* from Korea (Cho et al., 2022) and in other mussel species (Itoh et al., 2019; Vazquez et al., 2022). In the Mediterranean mussel, *Perkinsus* spp. trophozoites were mainly present into haemal spaces in the vesicular connective tissues that surround mussel digestive and reproductive organs, extending into the mantle. Haemocytes, probably granulocytes, perform phagocytosis accompanied by the formation of a capsule to guarantee pathogen elimination. In many cases, recruited granulocytes go through apoptosis, a mechanism that can be part of the mussel immune response or that could be performed by the parasite to evade host immune defence (Soudant et al., 2013). For example, *Perkinsus marinus* was found to modulate apoptosis of eastern oyster haemocytes challenged in vitro (Hughes et al., 2010).

Pathogen preference for specific tissues can have an impact on its life cycle and expansion into the host. For example, it may promote its persistence into the tissue, amplify transmission potential, and it can be associated with key virulent phenotypes. *P. olseni* is defined as BHR, as other *Perkinus* species such as *P. mediterraneaus*, *P. chesapeaki*, and *P. beihaiensis* presenting a Broad Host Range possibility, similarly to the trend observed for and *Marteilia refringens* that can affect both oysters and mussels (Arzul et al., 2014; Carella et al., 2010; Carrasco et al., 2012; Guo and Ford, 2016; Itoiz et al., 2022; Le Roux et al., 2001). Direct impact of transboundary animal diseases in aquaculture is a significant limitation for living animal's international trade, causing a high socio-economic impact on aquaculture exporting nations. Considering *Perkinsus* plasticity to many bivalve species, like other mollusc's pathogens, recognized international organizations should take this into account in the view of possible cross infection.

The RFTM assay was effective in detecting *Perkinsus* infection, and qPCR was the most sensitive in define pathogen presence. Both methods can be advised to detect a new infection and its prevalence in a given area. The result of phylogeny strongly suggests parasite transfer from clams of Asia and Australia, providing evidence that *P. olseni* from Italian and Spanish mussels are grouped together, but are genetically distant from other *P. olseni* described in Europe (Italy, France, and Spain). Moreover, haplotype network analysis revealed one haplogroup for mussels in Italy and another haplogroup for mussels in Spain, strictly linked to clams from Asia, America, and New Zealand.

The One Health approach acknowledges the connection of human, animal, and ecosystem health. TAADs are highly transmissible, spread very quickly through national borders, causing serious socio-economic consequences. More research is necessary for biosecurity and protection of valuable marine food resources for a growing human population. In the absence of proper surveillance of stocks, the movement of mussels could increase the risk of introduction into other areas and in the local natural population. In Europe, surveillance efforts regarding mollusc diseases is different between Member States and partly depends on the amount and the diversity of the shellfish production. Developing an environmentally friendly and competitive farming practice is a priority objective of the EU to reach high standards in terms of animal/human health and consumer protection. Mussel aquaculture accounts for 15 % of the global bivalve production. In Europe, Italy is the second main producer after Spain, with about 64,000 tonnes produced per year and considered one of the largest European markets with an average consumption of 120,000 tonnes per year (EUMOFA, 2019; Hough, 2022)

In our study, *P. olseni* prevalence of infection showed a slight pattern of seasonality, as it was higher in warmer seasons than in coldest. Literature reports that this tendency could be due to seasonal seawater temperature changes, as relatively higher temperature during warmer seasons may stimulate *P. olseni* proliferation.

The complex outcome of host–parasite interactions is regulated by different aspects including host biology and immune defence, pathogen virulence, and abiotic factors. Unfortunately, due to the lack of feedback from mussel farmers in Naples Bay, we have no data on possible mortality episodes in the area and how the presence of Perkinsosis could potentially be involved in the fluctuations of the population. Data of mortality are only present from Alfacs Bay, but we cannot conclude that its presence was the trigger of the event. Other studies are needed to define pathogen biology and virulence. *P. olseni* presence in mussels in more than one region of Europe raises possible concerns, considering the high economical value of mussels for the local aquaculture sector in both Italy and Spain.

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Chapter 5. Need for alternative diets in bivalve hatcheries.

The contents of this chapter were published in the journal Aquatic Living Resources.

Title: Molecular identification, life cycle characterization, and hatchery seed production of dwarf oysters from the Ebro Delta (Spain).

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Abstract

Dwarf oysters in the Ebro Delta are only observed growing on local pen shells, *Pinna nobilis* L., currently under extinction risk. The species identification of these populations is uncertain, given recent genetic classifications of dwarf oysters of *Ostrea stentina* species complex. Hence, the first objective of this study was to confirm the identity of dwarf oysters associated to *P. nobilis* in the Ebro Delta. Then, we aimed to assess the viability of hatchery and nursery production of *O. stentina* for potential conservation programs using diets based on a mix of live microalgae species or the commercial Shellfish Diet 1800®. Our results showed that COI sequences obtained were specific to *O. stentina*. Significant differences in the growth and survival of larvae between diet treatments were observed in 3 days. For the live diet, the larval period lasted from 15 to 22 days (299.2 ± 4 µm) whereas it took up to 36 days using the Shellfish Diet 1800® (280 ± 7.2 µm), with an overall mortality of 72.3 vs. 99.3%, respectively for each diet. Besides, no seed survival was observed for the commercial diet after a 10-day-fixation attempt. In contrast, ca. 16% of the individuals on the live diet that were allowed to settle reached the juvenile stage and could be released to the environment. Histological examination of obtained seed did not present any sign of disease and showed gonadal development for both sexes at ca. 6 months of age (16.1–19.1 mm). The growth curve obtained was fitted to a Schnute-Richards growth model which returned ages of up to 10 years for maximum local sizes of 45 mm. We conclude that although the Shellfish Diet 1800® is not an adequate diet for *O. stentina*, its hatchery production is feasible and might provide support to future conservation actions.

Key words: *Ostrea stentina* / DNA barcoding / Shellfish Diet 1800® / microalgae diets / Schnute-Richards growth equation / age at first maturity

1. Introduction

Oysters are widely distributed marine engineer species, capable of building reef habitat for entire ecosystems (Lenihan and Peterson, 1998) and providing services of great ecological and economical value including fisheries resources (Beck et al., 2011), effective attenuation against wave action and shoreline erosion (Meyer et al., 1997) and reduction of coastal eutrophication thanks to their ability to filter large volumes of water (Carmichael et al., 2012; Cerco and Noel, 2007). Unfortunately, over-harvesting, and pollution have contributed to globally reducing natural oyster habitats by as much as 85 percent over the last two centuries (Beck et al., 2011). Besides, different oysters' populations and species have been also severely affected by a range of parasitic, bacterial, and viral diseases, possibly enhanced by exposure to environmental stressors (Lenihan et al., 1999) and/or transfers of nonnative oysters for aquaculture and from ballast waters (Brenner et al., 2014). Among proposed solutions, a popular one has been the construction of artificial reefs to promote larval settlement on empty oyster shells or different man-made substrates (Goelz et al., 2020). Yet, most of such restoration efforts have been focused on enhancing oyster populations targeted by seafood markets to continue their exploitation (Coen and Luckenbach, 2000). In doing this, the conservation of other oyster species without commercial interest has been neglected. In addition, most of the hatchery production of bivalves is also focused on satisfying the demands of species of commercial interest (Helm et al., 2004), even when aimed at restoring natural banks to mitigate overharvesting (e.g., (Lodeiros et al., 2016; Loor et al., 2016)). To our knowledge, the only non-commercial bivalve species for which still infructuous efforts have been conducted to develop viable hatchery and nursery protocols is the critically endangered Mediterranean fan mussel, *Pinna nobilis* Linnaeus, 1758 (Trigos et al., 2018). Another key issue for restoration is the availability of local natural populations (Brumbaugh and Coen, 2009), whose shortage could potentially constrict hatchery production since a minimum genetic diversity is necessary in order to obtain viable populations in the long-term (Hughes et al., 2019).

The ostreid genus *Ostrea* Linnaeus, 1758 typically includes sequential hermaphrodites, viviparous species maintaining larvae within a brood chamber from days to weeks before releasing the swarm into the water column (Gray et al., 2019). Aside some economically important species such as *O. edulis*, *O. chilensis* and *O. angasi*, the genus also contains several less known and closely related species of dwarf oysters in the *Ostrea stentina*/*equestris*/*aupouria* complex (Hu et al., 2019). In particular, the *O. stentina* group appears to feature a geographic distribution across the Eastern Atlantic (from Morocco to the Iberian Peninsula) and Southern/Southwestern Mediterranean coast (Hu et al., 2019; Lapegue et al., 2006), although genetic confirmation is needed for most of the range. The species has been suggested to be a protandric hermaphrodite starting gametogenic development in February–March for females and in September for males (Ben Salah et al., 2012). It dwells on intertidal and shallow subtidal rocks and gravel beds or aggregated and low lying in the mudbank within marine and estuarine environments (Gonzalez-Wanguemert et al., 2004; Lapegue et al., 2006; Richards and Vitafinzi, 1982) and reach maximum sizes of ca. 4.5 cm (Rosique et al., 1995). In the Catalonian and Balearic region, fossil records of so called '*O. stentina*' range back to the Miocene and Pleistocene epochs (Cuerda et al., 1989; de Porta, 1987). In biological records from more recent periods, they have also been mentioned in numerous studies (e.g., (Aguilar-Amat, 1935; Altimira et al., 1981; Bonnin and Rodrígez-Babío, 1990; Tarruella and López, 2006)), but no population studies have been conducted, possibly because of the small size and lack of commercial value compared to other exploited species of oyster such as *Ostrea edulis* Linnaeus, 1758 or *Crassostrea gigas* Thunberg, 1793 (Beck et al., 2011). Yet, they might have an important role in the food web of marine ecosystems, enhance the availability of microhabitats, and contribute to positive effects on water quality. Hence, there is a need to overcome this overall lack of information on the genetic identity of dwarf oysters in order to assess possible alterations in the abundance of populations and associated ecological functions and allow addressing possible conservation efforts.

The same difficulties arise in Alfacs Bay (south Ebro Delta), where there is a historic record of the species (Brunet and Capdevila, 2005), but no population or genetic studies have been conducted. Currently, the presence of different native species of oysters, including *O. stentina* Payraudeau, 1826, *O. edulis* or *Anomia ephippium* Linnaeus, 1758, appears to be relegated to few isolated individuals settled on the spare hard substrate available within local seagrass beds of *Cymodocea nodosa* (Ucria) Ascherson 1870, but frequently they appear attached to the large shells of the Mediterranean fan mussel, *Pinna nobilis* which is currently in risk of extinction due to the expansion of the disease caused by *Haplosporidium pinnae* (Garcia-March et al., 2020). Previously to the disease, the pen shell population of the Alfacs Bay had been indicated as the second largest in the Mediterranean Sea, with over 90,000 individuals (Prado et al., 2014). Currently, the spread of the disease appears to be constrained by the salinity gradient induced by freshwater discharges from the local rice field agriculture (Prado et al., 2021b). Yet, a large part of the population has experienced mass mortality, thus compromising the presence of available substrate for the settlement of other organisms, including oysters. Present low abundances might be additionally influenced by the same confluence of factors such as pollution and disease also contributing to oyster reef decline at the global scale (Beck et al., 2011; Brenner et al., 2014; Lenihan et al., 1999), as well as to the local presence of the invasive blue crab, Callinectes sapidus Rathbun, 1896, which was first detected in the Alfacs Bay in 2012 (Castejón and Guerao, 2013) and currently supports a growing local fishery (López and Rodon, 2018). According to a recent study conducted with local commercial bivalves (*Crassostrea gigas* and *Mytilus galloprovincialis* Lamarck, 1819), only oysters larger than 5–7 cm are relatively protected from predation (Prado et al., 2020b), although experiments were conducted with detached animals, which ease manipulability compared to wild individuals (Lin, 1991).

In this context, given the current risk for the persistence of local dwarf oysters populations in the Ebro Delta, the main objectives of this study were: (1) to conduct a phylogenetic assessment to determine the genetic identity of local individuals relative to other dwarf oyster species within the *Ostrea* genus (Hu et al., 2019) and (2) to document the life cycle of the species and evaluate the viability of developing hatchery and nursery production techniques using different feeding methods. To this end, two different diets were tested on produced larvae, one based on live microalgae cultured in situ within experimental facilities, and other based on a popular commercial brand, Shellfish Diet 1800®, which has been successfully used in other species of bivalves such as the eastern oyster *Crassostrea virginica* Gmelin, 1791, the giant clam, *Tridacna noae* Röding, 1798, and the winged pearl oyster, *Pteria penguin* Röding, 1798 larvae (Rikard and Walton, 2012; Southgate et al., 2016; Southgate et al., 2017; Wassnig and Southgate, 2016). An assessment of health conditions was also performed on grown seed to determine whether individuals could be safely introduced in the wild. Finally, size increments were monitored for several months in order to have an estimation of the time required to attain maximum sizes observed in the field.

2. Materials and methods

2.1. Brood stock collection and conditioning

Brood stock collection was conducted on August the 19th 2020 on the southern shore of Alfacs Bay (south Ebro Delta), at a site hosting one of the few remaining populations of the Mediterranean fan mussel, *Pinna nobilis*, which provides substrate for the settlement of other bivalve species. Adult individuals (N = 2, ca. 45 mm in length) similar in appearance to *O. stentina* were transported to IRTA facilities attached to one of the pen shells Figure 5- 1, which was also captured as a part of a reproductive program to preserve the species (*Pinna* spat project). The pen shell and the two attached oysters were kept in an aerated 350 L truncated cone tank at 22 °C within an acclimatized room with 50% daily seawater renovation filtered and sterilized as for larval cultures (see later). Individuals were maintained with a daily dose of 15 X 104 cells/mL of *Tisochrysis lutea* El M. Bendif and I. Probert 2013 (T-ISO), 105 cells/mL of *Chaetoceros neogracilis* VanLandingham 1968, and 25 X 103 cells/mL of *Tetraselmis suecica* (Kylin) Butcher 1959. The strains were bought from the University of Santiago de Compostela and had the identification codes: ECC007 (*T. lutea*, 4–5 µm), ECC022 (*C. neogracilis*, 3–5 X 6–8 µm), and EC039 (*T. suecica*, 7–9 X 15–17 µm).

Figure 5- 1. Close up of a *P. nobilis* shell showing two attached adult individuals of *O. stentina* used for maturation.

2.2. Molecular identification of the oysters

A DNA barcoding strategy was used to identify the oyster species. DNA from 4 offspring from the two captive adults was extracted from a piece of fresh mantle tissue using the EZNA-Mollusc DNA extraction kit. A fragment of ca. 700 bp of the COI gene was amplified with the universal primers HC02198 (5'-taaacttcagggtgaccaaaaaatca-3') and LCO1490 (5'-ggtcaacaaatcataaagatattgg-3') (Folmer et al., 1994). PCR was carried out in 20 µl reactions with a final concentration of 2.5 ng/µl of template DNA, 0.2 mM of each dNTP, 1 µM of each primer, 1.5 mM of MgCl2, and 0.025 U/µl of Taq DNA Polymerase (Invitrogen) in the buffer supplied by the manufacturer. After 4 min at 95 °C, the PCR was subjected to 35 cycles of 1 min at 95 °C, 30 s at 56 °C, and 30 s at 72 °C, followed by a final 3 min step at 72 °C. PCR products were purified with PureLink Quick PCR Purification Kit (Invitrogen) and Sanger sequencing was carried out in both directions by the genomics service of the University of Valencia (Spain), using an ABI 3730XL automated sequencer and the same primers used for PCR amplification.

The sequences obtained were edited with Geneious Prime® 2019.2.3 and aligned with Clustal W 2.1 (Thompson et al., 1994) as implemented in Genious Prime. Seventeen additional COI sequences from different *Ostrea* species were downloaded from the GenBank database and added to the previous sequences in order to conduct a phylogenetic analysis with the software MEGA X (Kumar et al., 2016). These sequences were chosen among those used by Hu et al. (2019) to obtain a set of representative sequences of the different *Ostrea* taxa recognized in that study. Maximum likelihood composite (MLC) distances (Tamura et al., 2004) were estimated between pairs of sequences and the resulting matrix was used for the construction of a neighbour-joining (NJ) tree (Saitou and Nei, 1987). The statistical support of the tree nodes was estimated by a bootstrap test (Felsenstein, 1985) based on 1000 replicates.

2.3. Larval and seed culture

Once broodstock released the D-veliger larvae, water was filtered through a 40 µm sieve and subsamples counted under a stereomicroscope. A total of 700,000 larvae were obtained from one or both individuals (production of larvae was initially unintended but they were kept and cared for when they were released) attached to the pen shell: half of them (350,000 ± 14,530 D-veliger larvae) were fed the same three live microalgae that were used for brood stock conditioning, since these species are permanently produced at IRTA facilities. Mixed diets with different species of microalgae, including some of larger size such as *T. suecica*, have been used in other studies assessing the survival of veliger larvae in other species (Aranda-Burgos et al., 2014; Helm, 1977; Neo et al., 2013) and were considered as suitable for a first dietary assay with larvae of ca. 132 µm. The other half of the larvae were fed the Shellfish Diet 1800®, which is a mix of five marine microalgae: *Isochrysis* sp., *Pavlova* sp., *Tetraselmis* sp., *Thalassiosira weissdlogii*, and *T. pseudonana* (https://reedmariculture.com/products/shellfish-diet). D-veliger larvae were distributed in smaller 100 L truncated cone tanks at an initial density of 3.5 larvae mL^{-1}, resulting in only one tank per diet treatment. Although this fact poses the limitation of the absence of replicates for diet treatments, both tanks were located immediately adjacent to each other and exposed to the same conditions. Larvae and seed cultures were grown using filtered seawater through 1 µm and active C and irradiated with UV light (FSW) to minimize bacterial growth. Tanks were aerated and subjected to water change every 48 h. Temperature conditions were 22 °C and seawater salinity in our facilities ranged between 35 and 37 psu throughout the study period. For the live diet, microalgae cultures were prepared using the same filtered and irradiated seawater that was used for larval culture, under constant aeration and exposure to fluorescent light (47–67 µmol photons m^{2} s^{-1}). In addition, seawater used to start microalgae cultures for larval feeding was autoclaved in round 5 L flasks for 20 min at 120 °C to achieve further sterilization. The cultures were maintained in a thermostatic chamber at 22 °C illuminated with cool-white fluorescent tubes at light intensity of 150–200 µmol photons m^{-2} s^{-1} (Heinz Walz GmbH Universal Light Meter – ULM-500) under a 12 light:12 dark photoperiod, continuous mix of air and CO_2, and f/2 medium. During the first 10 days, larvae were feed every two days following each water change, with a total of 80,000 cells mL^{-1} at a ratio of 4 *T. lutea*: 2.5 *C. neogracilis*: 1.5 *T. suecica*, and then raised to 100,000 cells mL^{-1} (5:3:2 ratio) until the pediveliger stage. The Shellfish Diet 1800® consist of a mix of several marine microalgae: *Isochrysis* sp., *Pavlova* sp., *Tetraselmis* sp., *Chaetoceros calcitrans*, *Thalassiosira weissfloggi*, and *Thalassiosira pseudonana*. The diet was supplied in increasing daily doses following the instructions in the manufacturer web page (https://reedmariculture.com/collections/bivalve-hatchery-feeds). Both diets were supplied with a continuous feeding protocol, using a peristaltic pump controlled by a cycle timer that delivered the microalgae to larval rearing tanks every 60 min.

Once the postlarval stage was reached, production was transferred to a wider 100 L truncated cone tank in a closed, water recirculated system equipped with three settlement PVC cylinders with 150 µm mesh bottom and individual airlift systems. Each cylinder contained a fine layer of 300–500 µm sand made of crushed oyster shells.

Individuals on the live diet were fed with 120,000 cells mL^{-1} (5:3:2 ratio) for 12 days, and then the feed dose increased to 180,000 cells mL^{-1} for another 6 days, both at 48 h intervals. Then, seed started to be fed at increasing daily rates (10 days: 120,000 cells mL^{-1}; 7 days: 150,000 cells mL^{-1}; 7 days: 200,000 cells mL^{-1}; 12 days: 250,000 cells mL^{-1}; and 12 days: 300,000 cells mL^{-1}). For the Shellfish Diet 1800® doses were adjusted for size increases, following the same calculations described for the larval period, but stabilized when no growth was detected.

Remaining individuals that reached a size of ca. 5 mm (early November), were transferred to a larger 3000 L tank with an open-water system equipped with a 500 µm mesh bottom, PVC trays, with an airlift system connected to an automatic pumping system that delivered microalgae mix every 15 min. In mid-March 2021, ca. 25 % of the produced seed (the maximum that we were able to process) were carefully cemented to concrete construction blocks (ca. 60 × 25 × 35 cm) and experimentally released in Alfacs Bay, beside a mixed seagrass meadow (*Cymodocea nodosa* and *Zostera noltii* Hornem), located in front of IRTA facilities, to further assess survival and growth. The remaining 75 % was released into the same area or in Alfacs Bay, where brood stock were captured, but without attachment to a hard substrate to allow dispersion by currents into nearby areas. Only a small contingent of ca. 200 individuals were kept under vivarium conditions (open-water circuit) for a pre-fattening phase with supplied daily rations of ca. 4 % of body weight (1:3 T-ISO and *Tetraselmis suecica*).

2.4. Assessment of larval and seed sizes and number of surviving individuals

At each water change, a sample of larvae ($N \geq 20$ individuals) under each dietary condition was measured to the nearest µm from photographs taken with a camera coupled to a NIKON Eclipse TE2000-S microscope and to a computer featuring the Analysis 5.1 software for image processing. Later at early seed stages, individuals were measured to the nearest 0.1 mm with a NIKON SMZ 1500 stereomicroscope every 2–7 days. Finally, when individuals reached a manipulative size and transferred to the 3000 L tank, they were measured to the nearest mm once a month using vernier calipers.

The number of surviving larvae was counted from 1 mL aliquots ($N = 3$) taken from a 2 L measuring cylinder containing the homogenized larval pool at each water change. Once they were allowed to settle to sand particles, settlement success was assessed by counting the proportion of attached seed within randomly collected samples under the stereomicroscope. This was done on 8 occasions over a 20-day period in order to incorporate temporal variability due to the possible death of individuals during the settlement period.

2.5. Histological examination of produced seed

Twenty animals ranging from ca. 1.9 to 1.4 cm were collected once from the vivarium facilities at IRTA in February 2021, removed from the shell for fixation in Davidson solution, and preserved for at least 48 h at room temperature. Subsequently, they were dehydrated in ascendant alcohol series and embedded in paraffin blocks, 3 µm thick sections were cut with a Rotary Microtome (Bioptica, Italy) and stained with Carazzi's haematoxylin and eosin (HandE) and special stain Pas-BA pH 2.5 for detection of acid mucins (Mazzi, 1977). The slides were examined under a light microscope (Zeiss Axioscope) to assess the overall condition of the animal and possible presence of pathogens and diseases. Special attention was also paid to the presence of gonads and gametes in order to assess the age (and sex) at first maturity of the species. Samples for determining age at first maturity were collected during the first possible period of gonad development during the winter period and were not repeated because animals were already found to be mature.

2.6. Statistical analyses

Possible temporal differences in the size of individuals associated to the effect of diet were investigated with an independent samples two-tailed t-test for each sampling date. Similarly, differences in patterns of survival (i.e., estimated number of larvae in the tank) between the two diet treatments were also investigated with an independent samples two-tailed t-test but given the lower number of replicates per sampling date (N = 3 counts of each diet culture per date), all experimental period was considered together in order to increase the power of the test and obtain a more precise estimate. Student's t-tests were conducted in IBM SPSS Statistics v. 23.

The measured shell lengths of *O. stentina* across days of life in larval benthic stages were used to estimate Schnute-Richards growth function (Schnute and Richards, 1990). The Schnute–Richards model is a five-parameter model, given by the equation L(t) = L8(1+ δe−ktv)1/γ; where L(t) is oyster mean shell length at age t, L∞ is the asymptotic shell length, δ, ν, and γ are dimensionless parameters, and K is a relative growth rate parameter with units days–ν. Growth equation were fitted with R software version 4.1.1 through the minpack.lm 1.2-1 package.

3. Results

3.1. Molecular identification

Sequences of ca. 586 base pairs in length were recovered after editing the Sanger sequenced COI fragments. The four sequences were deposited with accessions OL404965-OL404968 in the GenBank sequence database and showed no polymorphism.

The alignment with the 17 *Ostrea* sequences downloaded from the GenBank database was over 650 bp in length, but only a fragment of 513 sites common to all sequences was used for phylogenetic inference (Figure 5- 2). The sequences obtained in this study joined other 4 sequences that belonged to the taxon *Ostrea stentina*, which were originally obtained by Lapegue et al. (2006) and Pejovic et al. (2016) from Atlantic localities in Morocco and northern Spain, respectively. The five sequences together formed a cluster with 100 % bootstrap support.

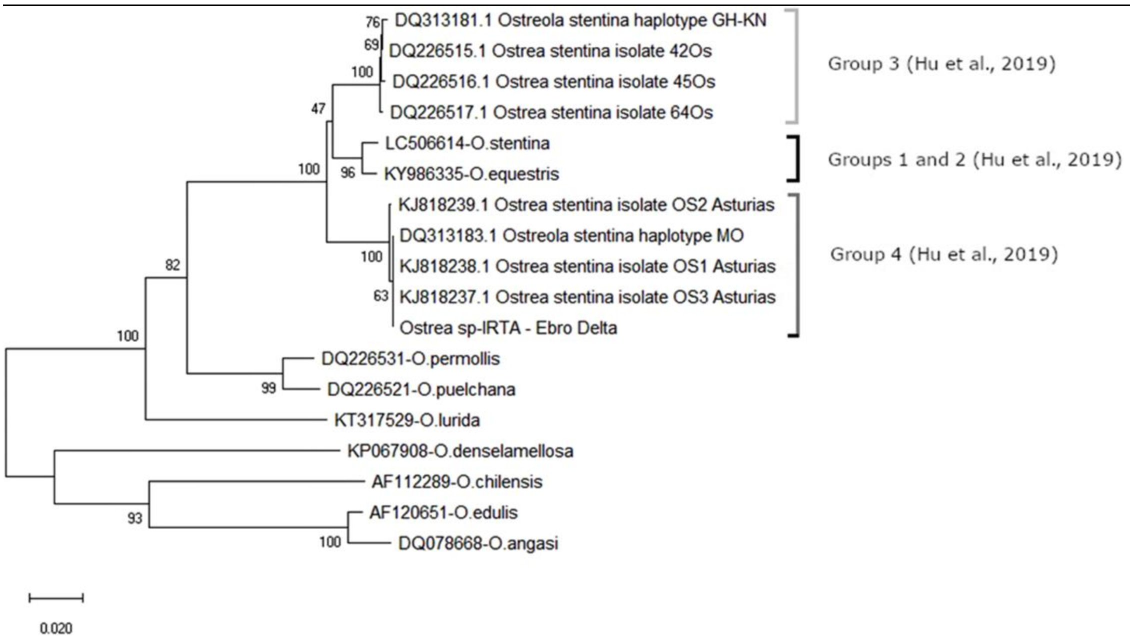

Figure 5- 2. Neighbor joining (NJ) tree based on Maximum Composite Likelihood (MCL) distances showing the phylogenetic affinity of the *Ostrea* sp. specimens used for this study (*Ostrea* sp.-IRTA - Ebro Delta) to *Ostrea stentina*. Numbers near nodes give bootstrap support (%). The inclusion in the different groups of dwarf oysters proposed by Hu et al. (2019) is indicated. Groups 1 and 2 are *O. equestris* and *O. aupouria* from close but divergent populations from the Americas and Asian Pacific, respectively. Group 3 is a new species *Ostrea neostentina* sp. nov. found in southeastern Spain (Mar Menor lagoon), eastern Tunisia, Hong Kong, and Japan, and group 4 is *O. stentina* occurring in northern (Avilés in the Atlantic Ocean) and northeastern Spain (Alfacs Bay in the Mediterranean Sea), Portugal, Morocco, and northern Tunisia.

3.2. Dietary effects on larval size and survival

D-veliger larvae of *O. stentina* were swarmed by brood stock at an initial size of 132 ± 1.1 µm (Figure 5- 3) two days after their arrival to our facilities and at a water temperature of 22 °C.

Results from t-test evidenced significant differences in larval size between the two tested diets only two days after the start of the culture, with the magnitude of differences increasing dramatically through time (Figure 5- 3 and Table 5- 1). For larvae fed with the live diet, most individuals settled at 302.8 ± 3.2 µm in a total of 15 days, although a small proportion was allowed to grow for an additional week (Figure 5- 3). In contrast, larvae under the Shellfish Diet 1800® took over a month to reach a size of only 280 ± 7.2 µm which was taken to attempt fixation (Figure 5- 3).

There were also significant differences in the survival rates of individuals between dietary treatments throughout the larval period (Student's t = –2.01, tcritic = 2.00, df = 52, P = 0.040) (Fig. 3B). Individuals under the live diet showed an overall mortality during the entire larval period of 72.3 % whereas mortality for individuals under the Shellfish Diet 1800® was considerably higher and reached rates of 99.3 % by the end of the pelagic larval period (Figure 5- 3).

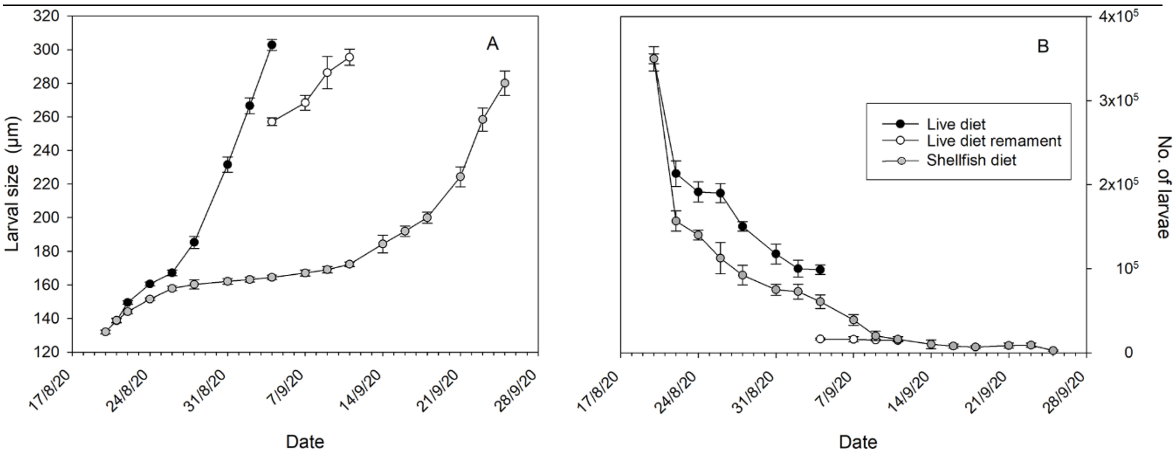

Figure 5- 3. (A) Larval size (first 15 days of life) of individuals fed with live microalgae vs. those fed the Shellfish Diet 1800®. For the live diet, the size of a group of larvae that remained in the tank while the majority was taken to fixation is also indicated. (B) Temporal changes in the number of larvae in of *O. stentina* feed with each type of diet. Error bars are mean standard error.

Table 5- 1. Results of two-tailed t-test for independent samples testing for differences between larval growth with the live microalgae diet and the Shellfish Diet 1800® at each experimental date. Significant differences are indicated in bold.

Date	***df***	***t* value**	**T_{critic}**	***P***
21/8/2020	37	0.123	2.035	0.9030
22/8/2020	37	2.740	2.026	**0.0094**
24/8/2020	37	8.017	2.032	**0.0000**
26/8/2020	61	-4.448	2.000	**0.0000**
28/8/2020	37	-15964.7	2.064	**0.0000**
31/8/2020	41	-14.394	2.020	**0.0000**
2/9/2020	37	-21.200	2.028	**0.0000**
4/9/2020	60	-35.145	2.000	**0.0000**
7/9/2020	56	-21.013	2.003	**0.0000**
9/9/2020	37	-11.834	2.306	**0.0000**
11/9/2020	37	-24.304	2.037	**0.0000**

3.3. Larval and post-larval development

Larvae under the live diet lost their D shape in 4 days (Figure 5- 4), started to show the umbo in 6 days (of 140.5 ± 1.1; Figure 5- 4), and all of them were umbonate 8 to 11 days after release (size range 167.1 ± 1.6 µm to 231.6 ± 4.5 µm; Figure 5- 4). During days 13 to 15th of life, larvae progressively increased in size until the pediveliger stage was reached (from 266.6 ± 4.7 to 302.8 ± 3.2 µm; Figure 5- 4E–F). At this size, some of the larvae started to show an "eyespot", the umbo leaned asymmetrically towards one side of the shell (Figure 5- 4F), and at times displayed the foot as they swam. Once in the settlement cylinders, individuals under the live diet increased their size from ca. 0.3 to 4.9–5.5 mm during the two first months (early September to early November) (Figure

5- 5A; Figure 5- 6A), with a rate of settlement success consistently above 70 % (Figure 5- 5B). In contrast, for individuals under the Shellfish Diet 1800®, no survival was observed after 10 days in the settlement cylinders. During the following two months (December to February), the seed under the live diet increased in size by 2.6-fold (Figure 5- 5A), and individuals started to attain juvenile traits such as a pattern of superimposed dark and clear bands on the right flat valve (Figure 5- 6B). Seed abundance counted on the 22nd of December 2020 yielded an estimate of 20,593 individuals. On the 19th of March 2021 individuals measured an average of 17.5 ± 0.7 mm and the estimated number was 15,900 individuals, ca. 16 % of the initial seed that were allowed to settle.

Individuals were introduced to a site close to IRTA facilities in the northern coast of the Alfacs Bay in March 2021 (3 days after biometric measures) but died ca. 2 months after. The site was chosen for logistic reasons but showed excess sedimentation over the concrete blocks that resulted on burial and anoxia. Surviving individuals during those two months (23rd of May and 26th of June 2021) yielded sizes of 18.2 ± 0.2 mm (N = 100) and 20.1 ± 0.2 mm (N = 31), respectively. Some additional animals (ca. 200 individuals) were, however, kept under vivarium conditions and random measures conducted on November the 11th, 2021 (N = 39 individuals), yielded sizes 21.1 ± 0.6 mm. The survival rate of these individuals was 88.5 % by February 2022.

The resulting Schnute-Richards growth equation between the measured shell length of individuals across days of life until November 2021 was:

$$L_{(t)} = 46.56(1 + 1.462e - 0.988t^{0.238})^{1/0.045}(R^2 = 0.93; P < 0.0001).$$

This results in age estimates of ca. 1.6 years to reach 30 mm, 3.5 years to reach 40 mm and up to 9.6 years for the theoretical maximum of 45 mm reported for the species.

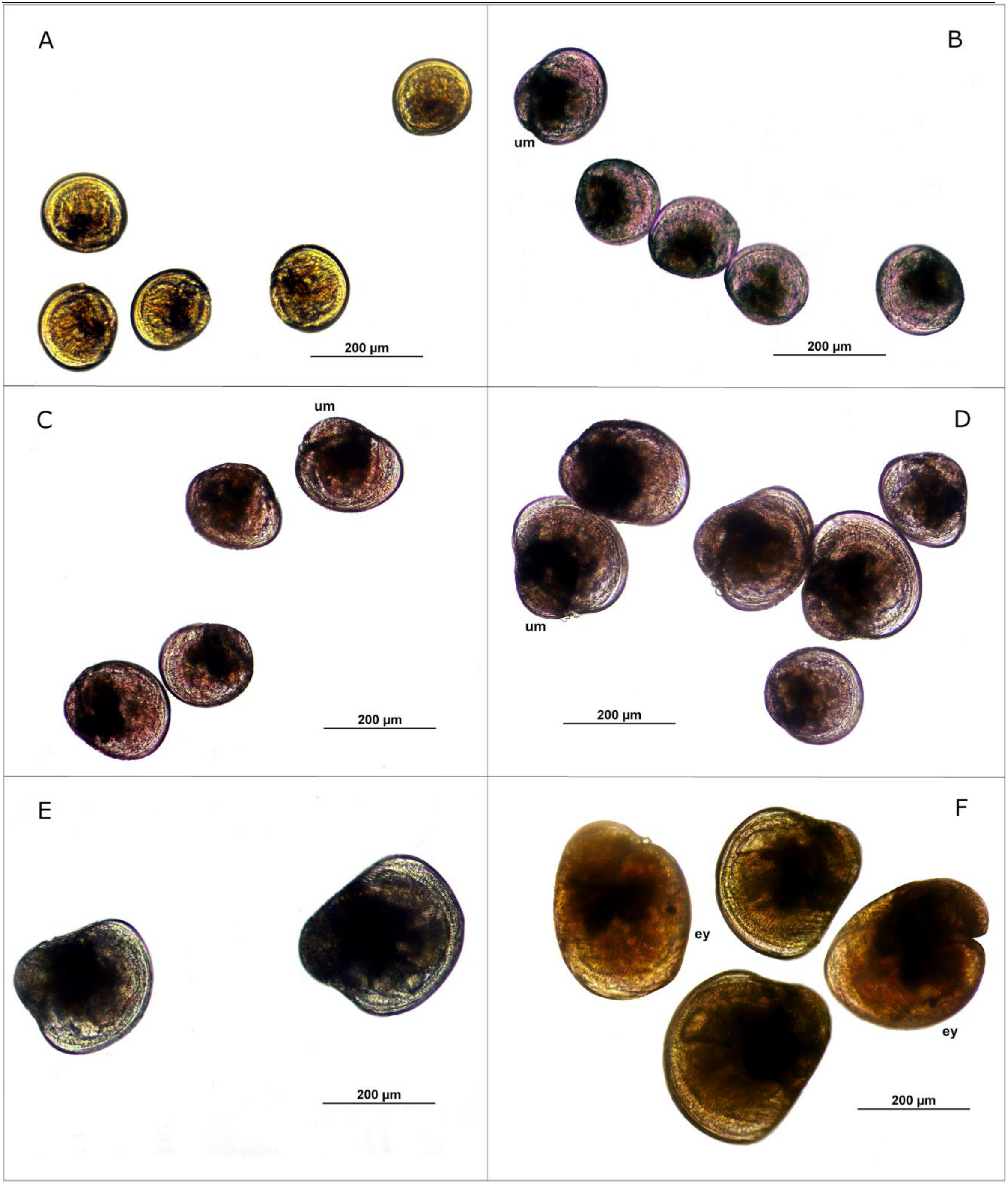

Figure 5- 4. Complete larval development of *O. stentina* raised with the live diet. (A) Veliger larvae with 4 days of life; (B) Larvae with 6 days of life starting to show the umbo (um); (C and D) umbonate larvae of 8 to 11 days of life; (E) early pediveliger (13 days of life); and (F) pediveliger stage, with some of the larvae showing an eyespot (ey) and the umbo leaning asymmetrically towards one side of the shell.

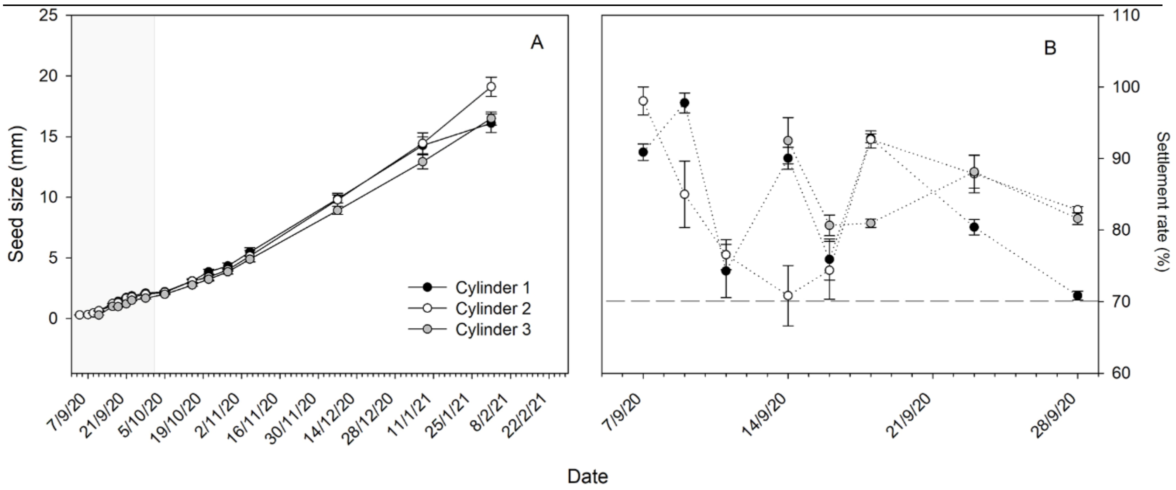

Figure 5- 5. (A) Seed size of *O. stentina* raised with the live diet in the three settlement cylinders. The settlement period is indicated in grey and corresponds to the time frame indicated in panel 5B. Larvae in cylinder 3 were set to settle 1 week later than those in cylinders 1–2. (B) Rates of settlement during the first 23 days of benthic growth. The 70 % success is indicated with a dashed line. Error bars are mean standard error.

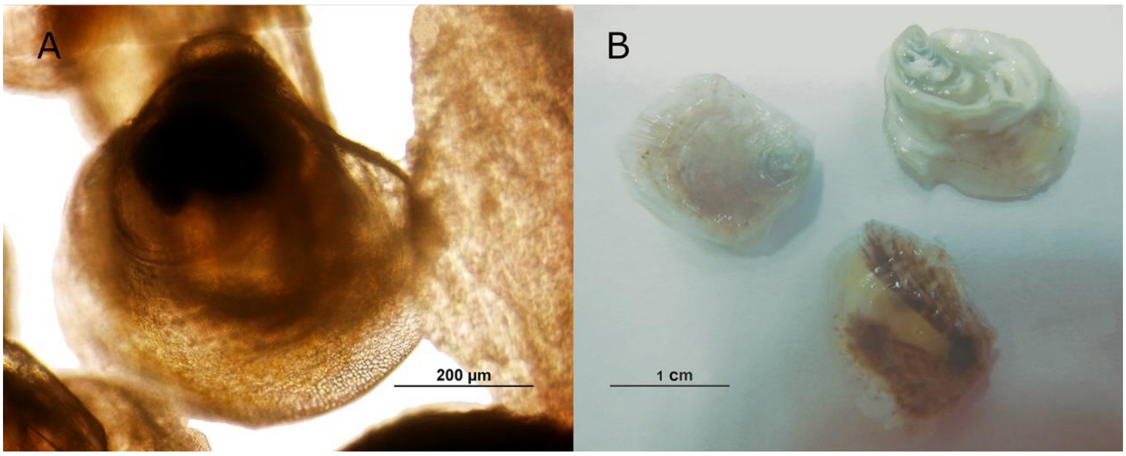

Figure 5- 6. (A) Seed of ca. 5 mm size in early November and (B) seed of ca. 14 mm size in early January already showing some juvenile traits such as a banded dark and clear pattern on the right flat valve.

3.4. Histological examination of produced seed

Light microscopy of the seed showed that young individuals of only 6 months of age were already sexually reproductive. Examined individuals showed hermaphroditism with alternating sex at the moment of sampling in February 2021. These individuals featured an apparent primary female development, with the ripe female gonad showing mainly vitellogenic oocytes attached to the follicle wall; at the same time, a developing male gonad was represented by growing follicles filled with germinative cells and spermatogonia present around its periphery (Figure 5- 7A and B). The adjacent digestive tissue showed a well-developed epithelium, indicative of an adequate feeding condition necessary for active growth and maturation (Figure 5- 7C). Overall, no evidence of a disease condition (i.e., presence of pathogens, and/or inflammatory responses) was observed. In fact, histological sections revealed an optimum development of the first line of immune defense constituted by mucous and granular cells in the mantle epithelium (Figure 5- 7D).

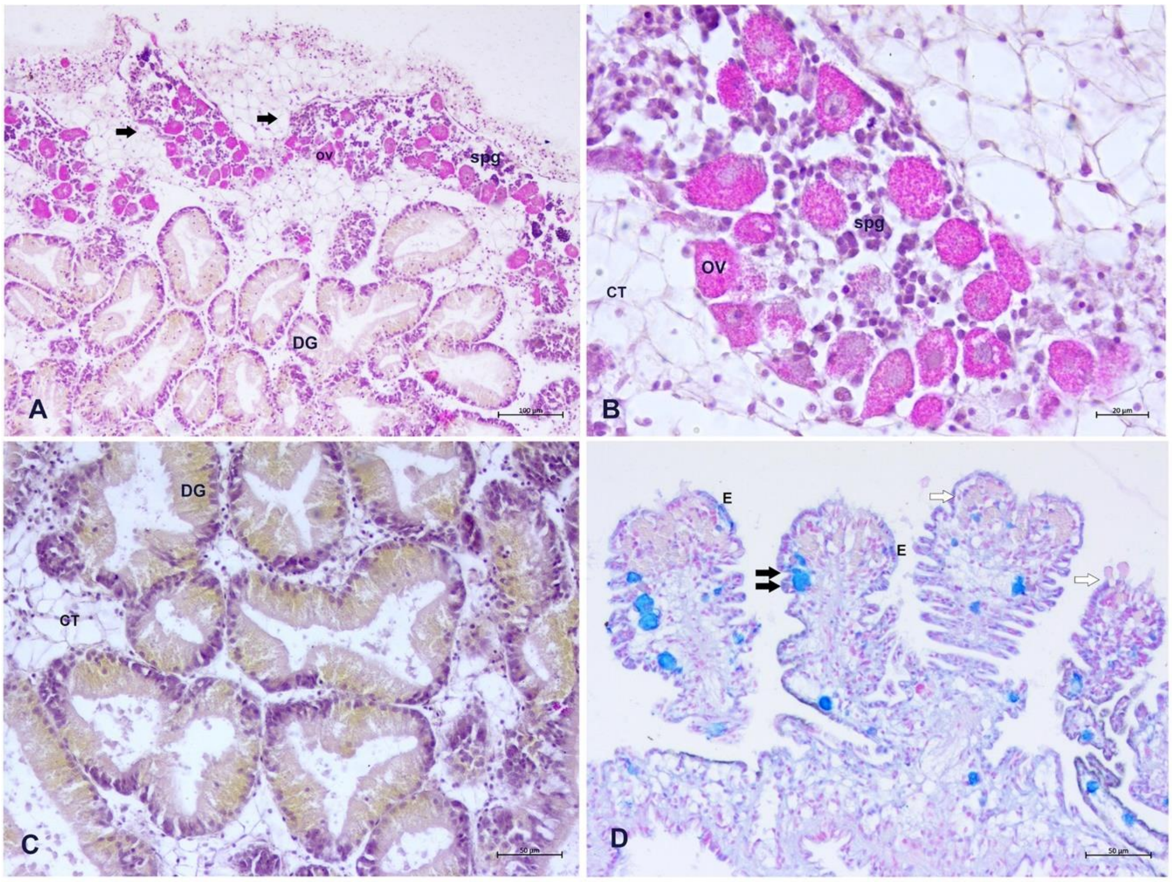

Figure 5- 7. Histology of different tissue of *O. stentina*: (A and B) visible hermaphroditic gonad with ripe female phase and developing male phase; vitellogenic ovocytes (Ov) and spermatogonia (Spg) are indicated; (C) digestive gland (DG) show a thick adsorbing epithelium sign of an active feeding; (D) Details of mantle epithelium (E) showing well developed mucous cells stained with acid alcian Blue Pas (black arrow) and eosinophilic granular cells (white arrow) part of the oyster first line of defense. CT: connective tissue.

4. Discussion

Our results confirm the presence of *Ostrea stentina* – according to the genetic classification of Hu et al. (2019)– in the Ebro Delta (Alfacs Bay). This finding contrast with results for dwarf oysters in the Mar Menor, the closest location (ca. 400 km) for which there is genetic information, which were classified as the new species *Ostrea neostentina* sp. nov. (Hu et al., 2019). This mismatch highlights the lack of knowledge on genetically verified geographic distributions and possible specific habitat requirements of these two Mediterranean species included within the *O. stentina* complex (Hu et al., 2019). According to current information, the *O. stentina* species (group 4 from Hu et al. (2019)) has been found in the Western (Alfacs Bay, Spain) and Southern Mediterranean Sea (Bizerte Lagoon, Tunisia), as well as in the Eastern Atlantic (from Dakhla Bay in Morocco to Sado and Mira estuaries in Portugal, and Avilés in the Bay of Biscay in Northern Spain). Yet, the finding of *O. stentina* in the Cantabrian Sea has been pointed as a non-indigenous species arrival associated with intense maritime traffic and disturbed environmental conditions (Pejovic et al., 2016; Rech et al., 2018). In former genetic research by Lapegue et al. (2006), authors suggested historical native presence range along the African Atlantic and Southern Mediterranean coasts but also include the northeastern coast of the Iberian Peninsula. Other works in the Catalonian and Balearic region based only on morphological descriptions (Aguilar-Amat, 1935; Altimira et al., 1981; Bonnin and Rodrígez-Babío, 1990; Cuerda et al., 1989; de Porta, 1987; Tarruella and López, 2006) also reported the presence of the species in the area, which is now genetically confirmed by our study. Regarding environmental conditions, genetically verified natural sites with *O. stentina* include sheltered bays with slightly brackish to marine salinity ranges and steady to strongly seasonal fluctuations in seawater temperature (El Asri et al. (2019), this study); and Atlantic estuaries with large freshwater supply and strong spatial gradients in salinity (Re, 1996), thus suggesting a wide plasticity in the physiological requirements. Even so, the species has not been yet genetically confirmed from hypersaline lagoons such as the Mar Menor.

Larvae obtained from brood stock attached to collected pen shell specimens were swarmed at an initial size of 132 ± 1.1 µm, which is in the lower range of that reported for other species in the genus, including *O. permollis* (108–127 µm), *O. edulis* (180–190 µm), *O. lurida* (169–185 µm), and *O. chilensis* (416–521 µm) (Buroker, 1985) and might be partly due to variability in the incubation period (from 3–9 days to 8 weeks, depending on the species; see Gray et al. (2019) and references therein. From that starting point, significant differences in the growth and survival of larvae fed live microalgae vs. those fed the Shellfish Diet 1800® diet become apparent only 3 days after the start of the experiment. The total duration of the larval period ranged from only 15 to 22 days (299.2 ± 4 µm) using the live diet to 36 days (280 ± 7.2 µm) using the commercial diet, with an overall mortality of 72.3 vs. 99.3 % by the end of the hatchery period, respectively for each diet. Although the robustness of our results is limited due to the lack of adequate replication, the conditions of both tanks were almost identical: they were positioned next to each other, got the water from the same source, and they were manipulated by the same person all the time. Therefore, it would be highly improbable that a potential pathogen would have developed in one tank and not in the other, although the possibility cannot be fully discarded. This makes the different diet provided to each tank the most reasonable explanation for the observed differences in growth and survival between tanks. Our findings contrast with those reported in other hatchery studies with bivalve larvae and suggest that the efficiency of the product could be partly dependent on the target species. For instance, Southgate et al. (2016) reported successful hatchery production of the winged pearl oyster, *Pteria penguin*, using Instant Algae® ("*Isochrysis* 1800®" and "*Pavlova* 1800®") with ca. 4.6 % of the larvae surviving to fixation (a total of 700,000 eyed pediveligers obtained), although no simultaneous comparisons were conducted with a traditional diet based on live microalgae. In another study, larvae of *Tridacna noae* were shown to ingest microalgae species from commercial concentrates using epifluorescence microscopy and displayed subsequent growth (Southgate et al., 2017). Authors also observed that veliger larvae displayed a distinctive temporal digestion of microalgae species, but this effect should not be responsible for the observed hatchery failure with *O. stentina*, since the product used (Shellfish Diet 1800®) consists of a mix of up to six different species. An alternative explanation might be the lack of a preliminary adjustment of the feeding ration for the species, as indicated for *P. penguin* larvae (Wassnig and Southgate, 2016). The diet was supplied following the manufacturer instructions, which in turn were based on FAO protocols for *C. gigas* and has been also successfully applied in *C. virginica* (Rikard and Walton, 2012). In fact, there is an abundant literature supporting the efficiency of Shellfish Diet 1800® in *C. virginica* in hatchery and nursery arenas as well as in adult conditioning (e.g., (Kroll et al., 2016; Pruett et al., 2021; Rolton et al., 2016) among others). Conversely, other species of bivalves have been shown to experience deficient physiological performance with commercial microalgae concentrates that support our present results. For instance, in recent experiments with the Manila clam conducted at IRTA with the same experimental diets and enhanced levels of replication (N = 3 and repeated three times) similar mortalities and decreased growth rates were obtained with the Shellfish Diet 1800® (unpublished results). Hernandis et al. (2022) compared scope for growth (SFG) in juvenile *Pinna rudis* Linnaeus, 1758 fed with Shellfish Diet 1800®, live cultured *Isochrysis galbana* Parke, 1949, and a 50/50% mix diet and found a negative energy balance (–5.67 $J\ g\ h^{-1}$), indicative of highly stressful conditions, that suggest lower acceptability and digestibility. Similarly, Prado et al. (2020a) and Prado et al. (2021a) used different species microalgae concentrates (Easy Reefs, Fitoplancton Marino) to feed juvenile *P. nobilis* and found 85 % lower growth than in controls based on live microalgae, along with almost double mortality rate associated to severe nutritional impairment and depletion of internal reserves. The reasons behind the failure of commercial concentrates in some bivalve species are unclear. Hernandis et al. (2022) performed analyses on cultured and commercial microalgal cells and showed that both had a similar organic content, but ash content was higher in commercial (19 %) than in laboratory cultured microalgae (5.3 %) which might result in decreased digestibility. It is also possible that the compounds used in commercial products to keep low bacterial loads and trigger flocculation (Camacho-Rodriguez et al., 2016; Knuckey et al., 2006) are not well tolerated by all bivalve species, thus causing detrimental side effects on the assimilation efficiency that might cascade to reduced growth and enhanced mortality. The very few individuals from the Shellfish Diet 1800® treatment that survived to reach a size of ca. 280 µm (ca. 0.7 %) and were transferred to fixation in settlement cylinders showed no survival after 10 days.

When live phytoplankton was used for hatchery production, larvae took 15 to 21 days in reaching the pediveliger stage for benthic settlement. This planktonic period appears to be highly variable across the *Ostrea* genus: 0–2 days in *O. chilensis*, 6–14 days in *O. edulis*, 10–23 days in *O. lurida*, and 30–33 in *O. permollis* (see review by Buroker (1985)) and might integrate species differences in growth rate and initial larval size, and the effect of environmental conditions. Larval survival with the investigated live diet (27.71 %) could be considered low compared with studies showing values from ca. 50 to >90 % in *O. edulis* (e.g., (Araya et al., 2012; Gonzalez-Araya and Robert, 2018; Robert et al., 2017)). In particular, the highest survival rates of up to 98 % were obtained for larvae fed with a mixed diet of *C. neogracilis* and *T. lutea* (1:1 cell volume) and maintained throughout the planktonic life at 25 °C using a flow-through rearing system (Robert et al., 2017). The progressive development of hatchery production techniques for this species since the late 1930s (Colsoul et al., 2021) suggest that the culture of *O. stentina* could also be improved using other diet formulations, daily rations, water temperature and/ or rearing system. In addition, since brood stock released larvae only two days after field collection, the potential effects of parental nutrition in progeny are unknown and could have limited larval development (Gonzalez-Araya and Robert, 2018). By the end of the planktonic period, a total of 97,000 late pediveliger larvae were allowed to settle in early and mid-September, with a mean of ca. 84 % experiencing settlement possibly favoured by temperature conditions (Robert et al., 2017) or by the presence to some unknown natural inducer such as bacterial biofilms or chemical cues (Mesias-Gansbiller et al., 2013). Yet only ca. 16 % of the late pediveliger larvae reached a juvenile stage and were still alive by mid-March 2021, evidencing that nursery production was also below values observed in other studies with *O. edulis* (ca. 28–40 % post-larval yields using mixed diets; Helm (1977); Araya et al. (2012). The obtained seed showed, however, a healthy condition, as evidenced by the presence of well-developed digestive tissue in histological examinations. Bacterial and protozoan diseases are common bottlenecks in bivalve hatcheries and in wild populations (see review by Paillard et al. (2004)), so their absence from the culture opens the door to their potential safe translocation to the natural media. Unfortunately, experimental introduction of juveniles cemented to concrete blocks was not successful due to excess organic matter and siltation from nearby agricultural drainage channels, causing observable burial on anoxic sediment which may lead to infiltration of exogenous bacteria and subsequent mortality (Coffin et al., 2021). Yet, the survival of some individuals for two months coupled with the maintenance of ca. 200 in nursery facilities allowed for fitting to a Schnute-Richards growth equation. Although growth rates might be slightly different in wild populations, this yield estimates of ca. 1.6 years of age for animals of 30 mm, 3.5 years for those of 40 mm, and up to 10 years to attain maximum sizes around 45 mm (Rosique et al. (1995); this study). This estimate of maximum age is coherent with that indicated for *O. edulis*, which may range from 10 to 14 years and with other species also reaching an age of 15 years or more, depending on preferential harvesting of larger animals (Lockwood and Mann, 2019; Richardson et al., 1993). The obtained growth equation was also similar in shape to that found for *O. edulis*, with the phase of asymptotic reached at ca. 6 years of age ((Richardson et al., 1993), this study). In fact, histological sections showed that individuals of about 20 mm featured hermaphroditism with alternating sex, which is consistent with observations reporting that *O. edulis* may produce eggs even in the first year of life (see review by Cole (1942)). Our results also showed a primary female development in the examined individuals, which is contrary to the protandric hermaphroditism often described for *O. edulis* (e.g., (Joyce et al., 2013; Kamphausen et al., 2011)) and for *O. stentina* (Ben Salah et al., 2012). Yet, distinctive groups of adult spawners, one first acting as females with considerably larger brood strength, and other acting first as males and producing a lower number of ripened eggs due to partially spent reserves have been also indicated for *O. edulis* (Cole, 1942). Besides, Maneiro et al. (2017) and Maneiro et al. (2020) observed the presence of simultaneous female and male gametes in the follicles at different maturation stages, suggesting that maturation is not a homogeneous process and that might be influenced by multiple factors including temperature, photoperiod, and nutrition. Hence, the tentative nature of this observations is stressed, since the evaluation of a larger number of individuals appears to be necessary to assess patterns of sex-change in flat oysters, particularly when extrapolating hatchery results to field patterns.

To conclude, our results reveal the presence of *O. stentina* in Alfacs Bay, which represents the only genetically confirmed location in the Western Mediterranean Sea so far. Individuals found in association with pen shells appear to be an adequate source of brood stock, although given the endangered status of this species (Garcia-March et al., 2020) the finding of individuals might become a limiting factor for restoration (Brumbaugh and Coen, 2009). Yet, we were able to grow larvae and obtain healthy spat, thus opening the door to the possible culture of the species for wild release, the best strategy for oyster conservation (Colsoul et al., 2021). Our results point that Shellfish Diet 1800® is not an adequate diet for the hatchery and nursery production of *O. stentina* despite positive results in other species (Rikard and Walton, 2012; Southgate et al., 2016; Southgate et al., 2017; Wassnig and Southgate, 2016). Further research is, however, needed to develop reliable protocols for production, particularly in terms of feeding regime (microalgae species and food ration), water temperature, and the implementation of flowthrough larval rearing systems to enhance growth and survival of larvae and spat to ca. 70 % higher rates reported in other species of flat oyster (Robert et al., 2017). The northern coast of Alfacs Bay does not seem to be appropriate for benthic reintroduction given the proximity to agricultural discharge channels, but the southern coast where the brood stock was obtained features a much lower influence of discharges (Prado, 2018), and could provide better results. Since *O. stentina* does not have a commercial interest, its wild reintroduction could constitute a new paradigm for assessing the efficiency of previous ‘restoration’ efforts (Coen and Luckenbach, 2000). Besides, an enhanced resilience of *O. stentina* to the disease caused by the protozoan parasite *Bonamia exitiosa* Hine, Cochennac and Berthe, 2001, has been reported (Hill et al., 2010), which might be an additional trait favouring the effectivity of this oyster species for ecological restoration and the recovery of ecosystem functions and services (Colsoul et al., 2021).

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Chapter 6. General discussion.

Shellfish aquaculture has an important role in the production of sustainable protein contributing to food security (Azra et al., 2021). Seafood, including shellfish is an important part of a healthy diet (Liu and Ralston, 2021). Bivalve aquaculture apart from producing food provides other benefits, the most important are nutrient removal and habitat structure (Barrett et al., 2022). Potential negative effects are depletion of phytoplankton/chlorophyll, organic enrichment of sediments, introduction of pathogens and invasive species (Barrett et al., 2022). Some potential negative effects, as depletion or enrichment of sediments can be overcome using good practices in the selection of the appropriate areas and defining their carrying capacity (Aquaculture Stewardship Council, 2019). The risk of the introduction of pathogens and invasive species can be overcome reducing the translocation of animals producing local bivalve seed.

Despite the demonstrated potential of bivalve aquaculture, the production in the Mediterranean Sea is stabilized between 130,000-150,000 tonnes, and decreasing in some countries (i.e., Italy). Bivalve aquaculture faces different threats that are limiting its development in the Mediterranean Sea: climate change, harmful algal blooms, water pollution, and appearance of new pathogens. Bivalve aquaculture is normally located in shallow nearshore areas which are highly impacted by global warming. The aquaculture industry in the Ebro delta is facing all these challenges trying to find solutions to maintain and even increase bivalve production. Our research has focused on: 1) finding solutions for the allocation of new shellfish growing areas for mussel aquaculture, 2) assessing HABs risk and trends in the Mediterranean Sea; 3) exploring the causes of unexplained mussel mortality events; 4) developing methodologies to facilitate the production of local seed.

1. New shellfish growing areas in open waters.

Summer marine heat waves in the Mediterranean Sea have produced mass mortalities of farmed bivalves (Lattos et al., 2022; Ramon et al., 2007), the allocation of new growing areas in deeper waters less exposed to these events needs the development and demonstration of tools to select the appropriate locations and their carrying capacity. Remote sensing offers and excellent opportunity to assess the suitability of coastal areas for bivalve aquaculture. We have demonstrated how the combination of *in situ* measurements and remote sensing can provide the necessary information to calculate the carrying capacity and suitability of coastal areas for mussel aquaculture in the Ebro delta.

The warming trend in the Mediterranean Sea is 0.035 °C/ year for the period 1992-2021 (Figure 6-1) which is more intense than the global warming trend (Urdiales-Flores et al., 2023). There is variability in the intensity of warming among different subareas of the Mediterranean basin (Pastor et al., 2020). The SST trends do not simply translate to equivalent trends in MWH characteristics, the drivers of MHW are therefore more complex, and vary between regions (von Schuckmann et al., 2022). The western Mediterranean Sea has experienced an increase in severity of marine heat waves, but the analysis of different datasets provides different results on the evaluation of severe marine heat waves important for the evaluation of their biological impacts (von Schuckmann et al., 2022).

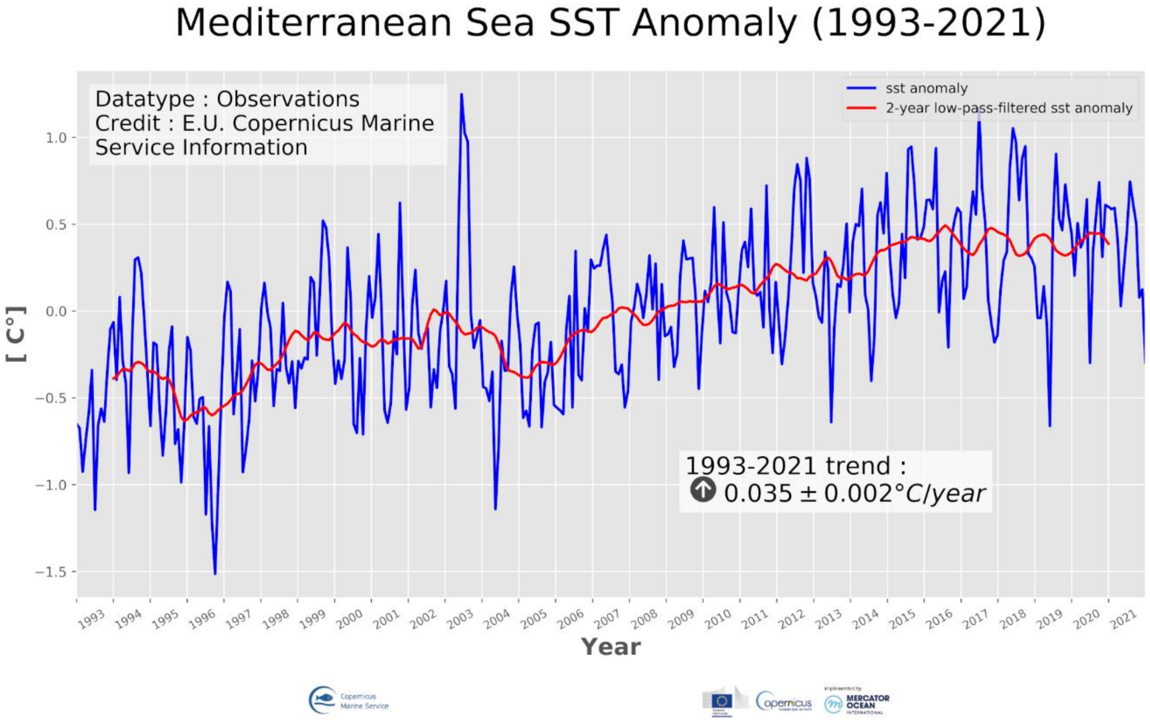

Figure 6- 1. Time series of monthly mean (blue line) and 24-month filtered (red line) sea surface temperature anomalies in the Mediterranean Sea during the period 1993-2021. Anomalies are relative to the climatological period 1993-2014 and built from the CMEMS SST_MED_SST_L4_REP_OBSERVATIONS_010_021 satellite product. https://doi.org/10.48670/moi-00268.

Another parameter of interest for the assessment of the suitability and the carrying capacity of shellfish growing areas is the concentration of chlorophyll-a as a proxy of phytoplankton biomass. In the Mediterranean Sea, there is a decreasing trend in the chlorophyll-a concentration -0.62 percent per year (Figure 6- 2). The decrease in the chlorophyll-a concentration is also observed along the Mediterranean coast of Spain where the highest chlorophyll-a concentration patches are located in the northwestern Alboran Sea and around the Ebro River mouth and near the shoreline (Gómez-Jakobsen et al., 2022). Remote sensing products are often unreliable in nearshore coastal environments and require a specific treatment (Cui et al., 2020; Gómez-Jakobsen et al., 2022; Tong et al., 2022). The algorithm developed in our study allows to estimate the chlorophyll-a concentration from Sentinel 2 images, for the coastal areas of the Ebro delta, with an accuracy higher than 70% using open-source tools and data (Fernandez-Tejedor et al., 2022). The maps created using this algorithm have been used to evaluate the suitability of the coastal areas of the Ebro delta and calculate their carrying capacity for mussel aquaculture using other open access data. The algorithm is less reliable under conditions of extreme weather events such as after the Gloria storm in 2020. The data obtained in the sampling cruises conducted during this study have been crucial for allowing the development of the algorithm. The availability of in situ data during extreme events is reduced due to the difficulties of conducting field sampling during adverse weather conditions. Our results show that the external areas of the Ebro delta can hold a significant production of mussels equivalent to 85-494 rafts from Alfacs Bay depending on the areas.

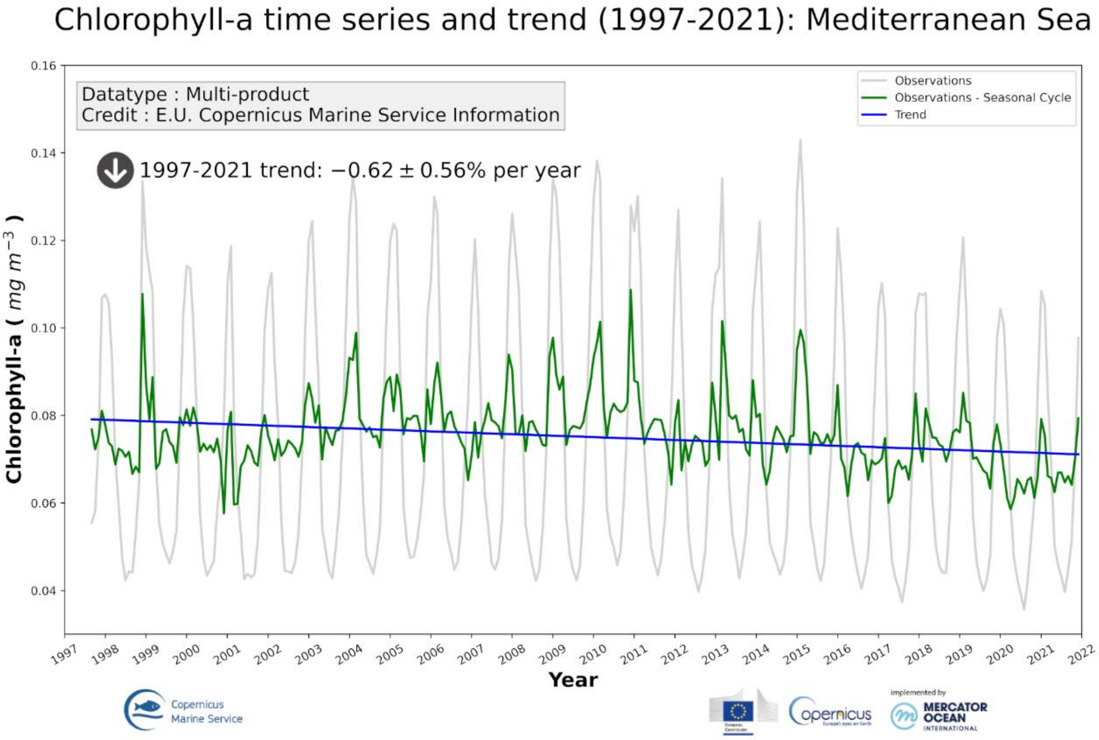

Figure 6- 2. Mediterranean Sea Chlorophyll-a time series and trend from Observations Reprocessing OMI_HEALTH_CHL_MEDSEA_OCEANCOLOUR_area_averaged_mean. https://doi.org/10.48670/moi-00259.

Other challenges that bivalve aquaculture is facing in relation to climate change are the increase in severe storms and sea level (Figure 6- 3). Recently, it has been found that changes in the Gibraltar ocean transport from the Atlantic Ocean into the Mediterranean Sea trigger sea surface height variability on average for the entire Mediterranean Sea basin, and these variations are anti-correlated to variations in the Atlantic Meridional Overturning Circulation (von Schuckmann et al., 2022). At the same time, the Meridional Overturning Circulation has a great impact on climate. Over the past 28 years, most extreme wave storms (i.e. the annual 99th percentile significant wave height) has increased in the entire Mediterranean Sea basin (von Schuckmann et al., 2022). The increase in severe storms is challenging for the design of the structures needed to build the farms outside the sheltered and protected embayments where it is now caried out.

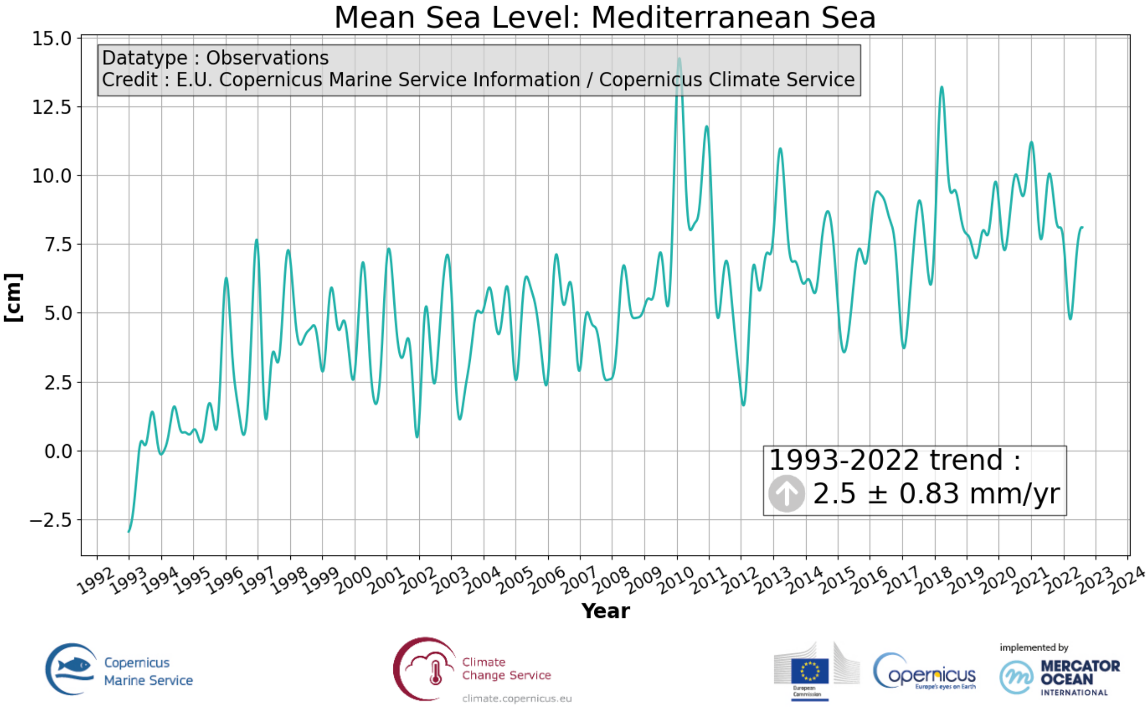

Figure 6- 3. Mediterranean Sea Mean Sea Level time series and trend from Observations Reprocessing. MEDSEA_OMI_SL_area_averaged_anomalies. https://doi.org/10.48670/moi-00264.

Forecasting the changes in the critical variables such as seawater temperature, chlorophyll-a concentration is needed to plan the future of bivalve aquaculture. At present, meaningful forecasts of changes in coastal primary production as a consequence of climate change are not available (Fly et al., 2015) to incorporate to mechanistic models. The role of bacteria, and how they are influenced by other stressors in shaping population and community-level responses to climate change and environmental stress, is frequently not included in the forecasts and this fact may underestimate the consequences of global climate change (Coffin et al., 2021). It is necessary to consider if the information provided by the different climate change forecast models is appropriate for the application in aquaculture for the site of interest, further processing may be required as for example a correction in the bias of seawater temperature (Falconer et al., 2023).

2. Trends in toxic phytoplankton in the Mediterranean.

Our evaluation of the available information on HABs from the Mediterranean Sea show that a high number of potentially toxic species are present and while seafood toxicity has been detected it usually occurs under regulatory levels and cases of intoxication are extremely rare. There is concern about the latitudinal shift in some species such as the species of the genus *Gambierdiscus* and its possible impact in seafood from the Mediterranean Sea. A high risk of high biomass blooms has been found affecting coastal areas devoted to tourism but no to aquaculture areas and a high risk of *Ostreopsis* blooms. The guidelines for the management of *Ostreopsis* blooms to protect human health in bathing waters along Italy, consider three levels based on *Ostreopsis* abundances in the water column: routine (≤10,000 cells L^{-1}), alert (10,000–30,000 cells L^{-1}), and emergency (>30,000 cells L^{-1}) (Funari et al., 2015). These alert levels have been found to be useful for the management of the risks of human intoxication though the consumption of shellfish exposed to these blooms (Accoroni et al., 2022). A study conducted in coastal areas around the Ebro delta showed that in spite of the occurrence of *Ostreopsis* cf. *ovata* blooms in areas close to the Ebro delta, this blooms do not occur in the areas where bivalve aquaculture is now carried out (Carnicer et al., 2015). *G. australes* from the Mediterranean Sea produces 44-methyl-gambierone MTX3 and MTX5, but no CTX precursors have been detected yet (Estevez et al., 2021; Estevez et al., 2020), and although the low CTX-like toxicities detected indicate that the potential risk of Ciguatera Fish Poisoning in the Balearic Islands is low (Tudó et al., 2020) their populations are likely to increase in the Mediterranean (Tester et al., 2020) and therefore the risk of CFP may increase in the future. Until now, this type of marine toxins is not considered as a threat for bivalve aquaculture. Closures of shellfish growing areas in the Mediterranean are usually related to the detection of okadaic acid during blooms of *Dinophysis* species or to the detection of saxitoxins during blooms of *Alexandrium minutum*, these species normally have a seasonal pattern. Monitoring programs of shellfish growing areas have been stablished in many Mediterranean countries, these monitoring programs generate time series of phytoplankton abundance and composition as well as in situ measurements of physico-chemical variables. The time series have been started to be used to forecast harmful algal blooms to species or genus level, for example for *Pseudo-nitzschia* and *Karlodinium* in the Ebro delta (Guallar et al., 2016) and for *Alexandrium* and *Dinophysis* in the Thau and Leucate lagoons (Bouquet et al., 2022).

The analysis of the timing of the productive period for the years 1998-2019 indicates that some general fertilizing mechanisms are acting over the whole Western Mediterranean, oscillations around the means of chlorophyll concentration of the productive period occurred synchronically in several subareas of the Spanish Mediterranean coast, indicating that the origin of this variability could be related with large scale climatic oscillations (Gómez-Jakobsen et al., 2022). These oscillations may have an influence not only on the total phytoplankton biomass but also on the phytoplankton species abundance and composition having a larger impact on shellfish farms located in open waters in comparison to nearshore more enclosed areas. At the basin level, the significant time lags found in the relation between SST and chlorophyll concentration are more than a year which could imply changes in the circulation regime (Katara et al., 2008). The impact of these oscillations at species level focusing on toxic species has not been investigated and could explain some trends in trends of toxic phytoplankton species. Civitarese et al. (2023) studied the impacts of the Adriatic-Ionian Bimodal Oscillating System on the planktonic communities from the Adriatic Sea using bibliographic information, they observed a significant impact on plankton distribution and abundance perturbating population patterns and introducing invasive species. They calculated that the saltier Levantine Intermediate Water (LIW) takes about 20 years to move from its formation area to the northwestern Mediterranean, therefore, the time lag between these oscillations and the impact on phytoplankton dynamics may be difficult to detect unless long time series are available. There are 235,638 records of dinoflagellate occurrences in the Mediterranean Sea registered at OBIS, and among them the time series of phytoplankton data from the Ebro delta (Fernández-Tejedor et al., 2020). In the future, forecast modelling based on long-term time series of phytoplankton in combination with in situ measured physico-chemical parameters and meteorological data from different geographical locations could be employed to simulate future scenarios, at species level, under climate change conditions.

3. New pathologies.

Our results represent the first report of *P. olseni* in *M. galloprovincialis* from the Mediterranean Sea, this parasite produces Perkinsiosis disease that affects many bivalve species. The prevalence of infection showed a seasonal pattern higher in warmer than in colder seasons. Higher temperature may stimulate its proliferation. Mussels, are able to be exposed to a high number of potential pathogens without getting infected and without developing an elevated inflammatory response (Figueras et al., 2019). Mass mortality events of mussels occurred in the Netherlands (Capelle et al.,

2021) and northern Brittany (Charles et al., 2020) no single putative causes could be identified, mortalities were attributed at a combination of factors. In the Thermaikos Gulf (Greece) the effect of marine heat waves combined with *M. refingens* infection led to devastating mortalities of *M. galloprovincialis* (Lattos et al., 2022). *M. galloprovincialis* has been considered a resistant species to *Vibrio* infections, but Balbi et al., 2019 found that *V. coralliilyticus* could represent a potential threat to mussel aquaculture as emerging pathogen. After the mass mortality events of farmed mussels observed in France and the Netherlands between 2014-2016 and 2019, Lupo et al 2021 reviewed the risk factors for mussel mortality. Their review raised the recommendations of developing standardised methodologies to estimate mussel mortality; exploring the combined effects of multiple factors on the mussel mortality risk including the life history of the mussel population and the impact of husbandry practices; and finally, the development of screening tools to capture multiple pathogens and pollutants in routine. Recent mussel mortalities in the Ebro delta that occurred during times of the year when seawater temperature was not limiting for the survival of *M. galloprovincialis* were localised in some of the farms. The mussel mortality event in June-July 2020 in Alfacs Bay occurred in a mussel farm that was the most exposed to pollution from the drainage channels discharging in Alfacs Bay, we cannot conclude that the presence of *P. olseni* alone triggered the disease, the combined effect of multiple factors including chemical pollution should be explored in future mortality events. The polymicrobial character of mussel disease has been demonstrated by Ben Cheikh and Travers (2022) in laboratory experiments in which they exposed *Mytilus edulis* to the bacterial pathogen *V. splendidus,* they observed that the disease results from a collaboration between external pathogens and pathobiont bacteria from the mussel microbiota. Their results provide a new insight into marine epizooties and therefore the study of the microbiome to detect dysbiosis will provide information on the infectious process and diagnosis. The integration of studies on the change of the host microbiome with environmental factors together with other technics, including microscopy, (epi)genetics, and immunology, will enable a better management of shellfish diseases (Paillard et al., 2022).

4. Need for alternative diets in bivalve hatcheries.

Mass production of microalgae to feed bivalve larvae and spat in hatcheries may be a limiting factor, the use of alternative diets instead of live phytoplankton could facilitate the production of bivalve seed. Phytoplankton concentrates can be stored and used when needed instead of upscaling and maintaining high volumes of live phytoplankton cultures. Some of these products require to be stored frozen or cold, others can be stored at room temperature. The nutrition of bivalve larvae has not received as much attention as the that of fish or crustacean larvae for which artificial diets are available (Robert and Trintignac, 1997). One of the main problems of using these products is to detach individual cells from the aggregates formed during preparation and storage. Shaking and blending have been proposed as previous treatments to separate individual cells from the aggregates and provide the right size of particles to bivalve larvae (Aji, 2012). These concentrates have been used in long-term experiments to feed adults of *Mytilus edulis* and the results showed a decrease in condition index that authors attributed to the fact that the mussels had food available only during part of the day (Mackenzie et al., 2014). Products based on algal concentrates are preferred by some researchers instead of live phytoplankton to test the effects of other parameters reducing the variability that using live phytoplankton could force in the experiments with bivalve larvae (Venkataraman et al., 2019). Marshall et al. (2010) reviewed how different types of food affected bivalve larval growth and survival, they found that although in general better growth and survival was obtained with life phytoplankton, concentrated products offered adequate growth rates for *C. gigas*. A different strategy, microencapsulated diets have been tested in *Ostrea edulis* spat resulting in higher survival of the spat but lower growth in comparison to spat fed with live algae (Willer and Aldridge, 2019).

5. The future of shellfish aquaculture in the Mediterranean Sea

The Mediterranean Sea is characterized as a low-productivity ecosystem, <150 gC m^{-2} $year^{-1}$, it is a mesotrophic sea, where many parts are oligotrophic and ultraoligotrophic (Stambler, 2014). This low productivity is considered a limitation for bivalve and seaweed aquaculture. Some coastal areas near river outflows have the highest chlorophyll concentration, and although the temporal trend in chlorophyll concentration across the basin is negative, a positive trend has been found in some local regions such as the Balearic Islands, the Ligurian–Provençal basin, the Rhodes Gyre region, and off the Nile River delta (Colella et al., 2016). Bivalve and seaweed aquaculture have been proposed as an opportunity to recover ecosystems from eutrophication. The Mediterranean Sea is ranked among the top 20 marine ecoregions that could benefit from the development of shellfish aquaculture where the score on the environmental and social challenges that shellfish aquaculture could combat is very high (Theuerkauf et al., 2019). In the Mediterranean Sea, the combined effect of excessive fishing pressure and changes in the primary productivity have altered the marine ecosystem over time, especially reducing the proportions of top predators and larger fish and increasing the abundance of groups at lower trophic levels since the 50s (Piroddi et al., 2017). Aquaculture is often considered as a stressor for wild fish stocks pressure mainly due to the harvesting of forage species to produce fishmeal (Bogmans and van Soest, 2022). Bivalve farming provides valuable benefits for the ecosystem in relation to nutrient removal and habitat provision (Barrett et al., 2022). Another ecosystem benefit is related to the carbon balance, mussels were found to be slight carbon sinks (Bertolini et al., 2023) at 12 Mediterranean sites. As an example, mussel farming in Sacca di Goro (Northern Adriatic Sea, Italy) has a net carbon capture capacity of 91 g CO_2/kg of fresh product (Tamburini et al., 2022). Bivalve aquaculture may have negative effects when the carrying capacity is exceeded, bivalves compete for phytoplankton with wild populations of filter feeders. The translocation of farmed bivalve populations is a risk for the introduction of invasive species and pathogens (Brand et al., 2022; Costello et al., 2021; Di Blasio et al., 2023; Geburzi and McCarthy, 2018). Ballast water is another introduction route for aquatic invasive species. The blue crab *Callinectes sapidus* has been introduced to the Mediterranean probably through ballast water (Holthuis and Gottlieb, 1955).The northern Adriatic Sea has been invaded by *C. sapidus* and at present, it is threatening the clam farming industry of the Po River delta in the Veneto and Emilia-Romagna regions, which normally produces about 15,000 tonnes of clams per year (Sabelli, 2023). The use of coastal ponds to grow clams avoiding the blue crab predation could help to maintain this industry. In addition, these ponds may be used for farming a combination of species presenting complementary ecosystem functions which has advantages for the use of natural resources. In this sense, integrated multi-trophic aquaculture (IMTA) which combines de cultivation of fed species with organic extractive species and inorganic extractive species (Boyd et al., 2020) could reduce the impacts of fed aquaculture. Bivalve aquaculture integrated in fish farms could help to mitigate the footprint of finfish farms (Papageorgiou et al., 2023) reducing the eutrophication. Bivalve farms located near finfish cages can benefit from the increased phytoplankton production but could be more exposed to predation by wild fish since remarkable aggregations of wild fish are often observed beneath sea cage farms (Akyol et al., 2020). Predation of farmed bivalves by seabream in some Mediterranean areas such as in Prevost Lagoon, southern France (Richard et al., 2020) and the Adriatic Sea (Šegvić-Bubić et al., 2011) may produce important losses. The use of special gear to protect farmed bivalves against predation will be needed in sites presenting a high risk of predation as bivalve farms located near fish cages. The development of innovative aquaculture solutions (IMTA, offshore, multi-use) and effective early warning systems to manage food security risks have been identified as needs for a healthy and resilient Mediterranean Sea during the Ocean Decade preparatory phase (Cappelletto et al., 2021).

Some criticisms to the development of bivalve aquaculture rise from the low yield of edible meat in bivalves (17 %) in comparison to finfish (87%), limited demand in some markets and the “appropriation of oceanic space” (Belton et al., 2020). Promotional campaigns to raise consumer awareness of the positive impact of bivalve aquaculture could help to increase demand for certified products (Xhakollari et al., 2023). Aquaculture site selection and site management facilitated through integrated coastal zone management (ICZM) and the use of the ecosystem approach to aquaculture

(EAA), were already identified, more than 10 years ago, as a priorities for the sustainability of aquaculture in the Mediterranean Sea (Rosa et al., 2012).

6. General conclusions

1) The algorithm developed using the C2XC processor for atmospheric correction and applying the REB1 band ratio with a 2nd polynomial fitting allows to estimate the chlorophyll-a concentration from Sentinel 2 images with an accuracy higher than 70% using open-source tools and data.

2) The application of the Dynamic Energy Budget theory using multispectral remote sensing imagery allows to rank the suitability of the different areas for shellfish aquaculture. It also allows to determine the periods of the year with favourable and unfavourable conditions for shellfish growth.

3) The external areas located close to the Ebro delta embayments can hold a significant mussel production that would allow the reduction of mussel mortality during summer months due to the lower seawater temperature in these areas.

4) The Mediterranean Sea hosts a high number of potentially toxic species, many of which have a wide distribution across its coastal waters. The cases of intoxication are extremely rare, marine toxins although present usually they are detected below the safety limits.

5) Large amounts of palytoxin-like substances accumulate every summer along the rocky Mediterranean shores due to *Ostreopsis* blooms. Although sea urchins and wild mussels inhabiting those environments at time accumulate those toxins to considerable levels, no cases of seafood intoxication have occurred so far.

6) There is relatively low risk deriving from toxic blooms but higher risk from high biomass blooms affecting the aesthetic qualities of coastal areas devoted to tourism in the Mediterranean Sea.

7) No clear trends in occurrence nor expansions emerge for either toxic or HB-HABs in the Mediterranean Sea.

8) *Perkinsus olseni* in *Mytilus galloprovincialis* in the Mediterranean Sea is reported for the first time, it is a parasite that generates an inflammatory disease, it is highly transmissible, spread very quickly through national borders, causing serious socio-economic consequences. *P. olseni* presence in mussels in more than one region of Europe raises possible concerns, considering the high economical value of mussels for the local aquaculture sector in both Italy and Spain.

9) The Ray's Fluid Thioglycollate Medium (RFTM) assay was effective in detecting *Perkinsus* infection, and qPCR was the most sensitive in define pathogen presence. Both methods can be advised to detect a new infection and its prevalence in a given area.

10) The result of phylogeny strongly suggests parasite transfer from clams of Asia and Australia, providing evidence that *Perkinsus olseni* from Italian and Spanish mussels are grouped together, but

are genetically distant from other *Perkinsus olseni* described in Europe (Italy, France, and Spain). Moreover, haplotype network analysis revealed one haplogroup for mussels in Italy and another haplogroup for mussels in Spain, strictly linked to clams from Asia, America, and New Zealand.

11) In our study, *Perkinsus olseni* prevalence of infection showed a slight pattern of seasonality, as it was higher in warmer seasons than in coldest. Literature reports that this tendency could be due to seasonal seawater temperature changes, as relatively higher temperature during warmer seasons may stimulate *Perkinsus olseni* proliferation.

12) We cannot conclude that the presence of *Perkinsus olseni* was the trigger of the event. Other studies are needed to define pathogen biology and virulence.

13) Significant differences in the growth and survival of oyster larvae fed live microalgae vs. those fed the Shellfish Diet 1800® diet were apparent only 3 days after the start of the experiment. The very few individuals from the Shellfish Diet 1800® treatment that survived to reach a size of ca. 280 µm (ca. 0.7 %) and were transferred to fixation in settlement cylinders showed no survival after 10 days. When live phytoplankton was used for hatchery production, larvae took 15 to 21 days in reaching the pediveliger stage for benthic settlement. Our results point that Shellfish Diet 1800® is not an adequate diet for the hatchery and nursery production of *Ostrea stentina* despite positive results in other species.

7. Fundamental gaps of knowledge and recommendations for future research.

The algorithm developed in our study to estimate chlorophyll-a concentration from Sentinel-2 tends to overestimate chlorophyll-a concentration in conditions that may occur after strong winds or storms. The development of a switching multi-algorithm requires the accurate definition of the optical properties, pixel by pixel and in situ data covering all different scenarios. The logistics of in situ sampling under extreme conditions may be a limiting factor for this development as occurred in our study.

We have shown how small changes in renewal time have an important impact in the carrying capacity of an area for mussel aquaculture. The carrying capacity model used in our study applies a constant renewal time for the two bays, but renewal times are not uniform inside the embayments, hydrodynamic models such as the Regional Ocean Model System (ROMS) coupled to a biogeochemical model nutrient-phytoplankton-zooplankton-detritus (NPZD) will provide more detail on the carrying capacity of the embayments.

-The integration of hydrodynamic and biogeochemical models in carrying capacity models will provide more accurate evaluations of the different coastal areas for bivalve aquaculture.

There is a marked west-east and north-south gradients in the knowledge of HABs in the Mediterranean Sea with long sections of the coast with scarce or no information available. The development of monitoring programs in these areas and the maintenance of a Mediterranean network of experts on HABs will reduce these gaps of knowledge. We have created a database built on bibliographic information; the database available at the Ocean Biodiversity Information System (OBIS).

-HABs forecast models at species level for regional areas using artificial intelligence in the future will benefit from open access data included in international databases that are incorporating at present long-term time series. The use of dataset from different locations in a regional area will be a tool to improve HABs forecast models.

Changes in climate are already changing hydrographical features in the Mediterranean Sea and are already driving variations in microbial populations in the plankton and the benthos. Further studies on HABs patterns and trends will need to incorporate climate change predictions.

-The application of the two first recommendations have a limitation related to the reliability of climate change forecast at local level at the accuracy needed for the biology of the species. There is a need for the improvement of climate change forecast models at local scale, being the seawater temperature and the chlorophyll concentration the variables of highest interest. Long-term forecast models for seawater temperature and chlorophyll concentration may be improved through ocean reanalysis at local/regional level.

There is scarce knowledge on the potential negative effect of *Perkinsus olseni* on *Mytilus galloprovincialis*, considering pathogen prevalence, intensity, and aspects of the inflammatory response. More studies are needed to assess the impact of Perkinsiosis in this new host. The presence of *Perkinsus olseni* should be investigated in other mussel mortality events.

-The study of unexplained mussel and oyster mortality events will benefit from the integration of microbiome and pathobiome studies as well as pollutants since these events are turning to be considered multi-factorial.

At present the use of the available commercial alternative diets in bivalve hatcheries is not recommended as a substitute of live phytoplankton. These products offer a good mixture of microalgae species embedded in a matrix. The compounds used in this matrix seem not to be well tolerated, reducing the assimilation efficiency of these commercial alternative diets.

-The composition of phytoplankton concentrates needs to be improved mainly in relation to its final presentation in the form of single cells and not aggregates. The methods used in the harvesting of phytoplankton cultures should consider the need of reducing cell aggregates.

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Annex

Supporting information of Chapter 1.

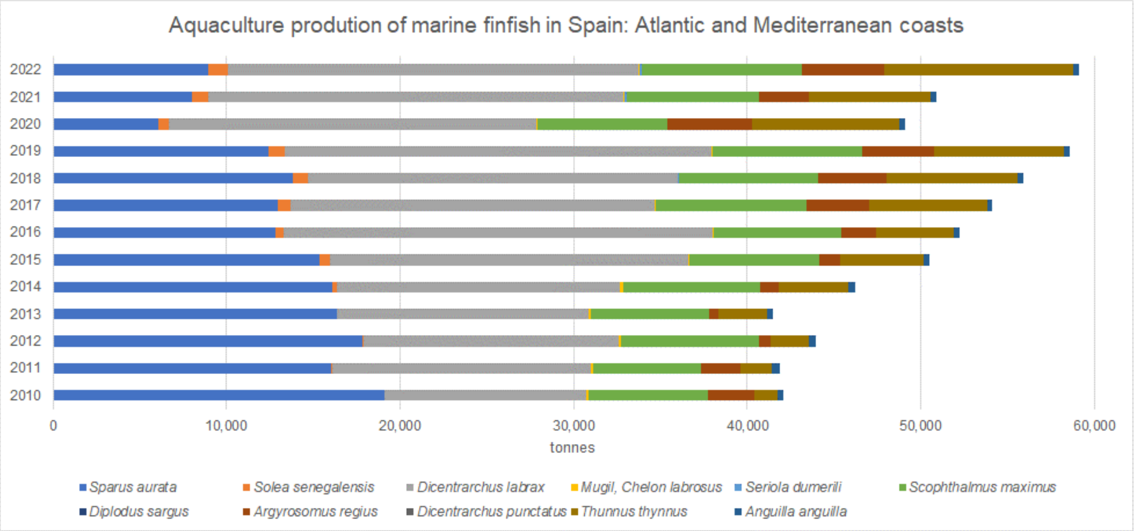


Figure 1S- 1. Aquaculture production of marine finfish in Spain: Atlantic and Mediterranean coasts (JACUMAR, 2023).

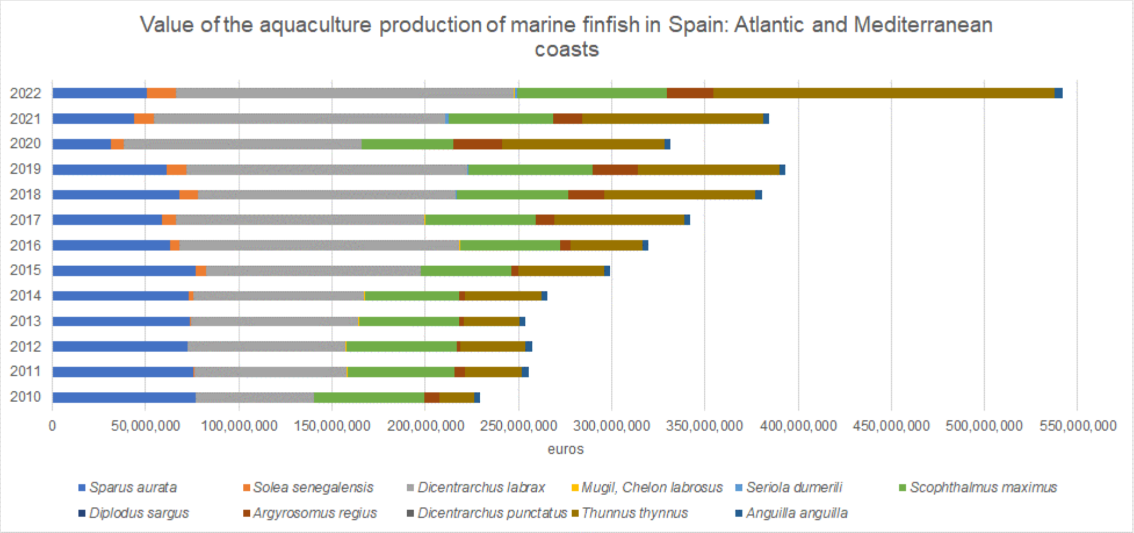


Figure 1S- 2. Value of the aquaculture production of marine finfish in Spain: Atlantic and Mediterranean coasts (JACUMAR, 2023).

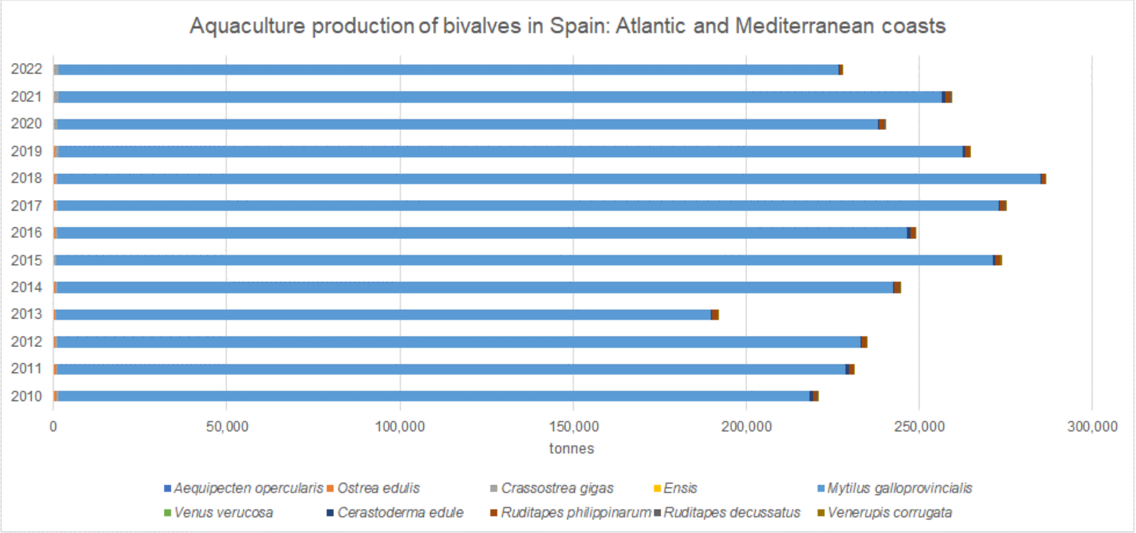


Figure 1S- 3. Aquaculture production of bivalves in Spain: Atlantic and Mediterranean coasts (JACUMAR, 2023).

Supporting information of Chapter 2.

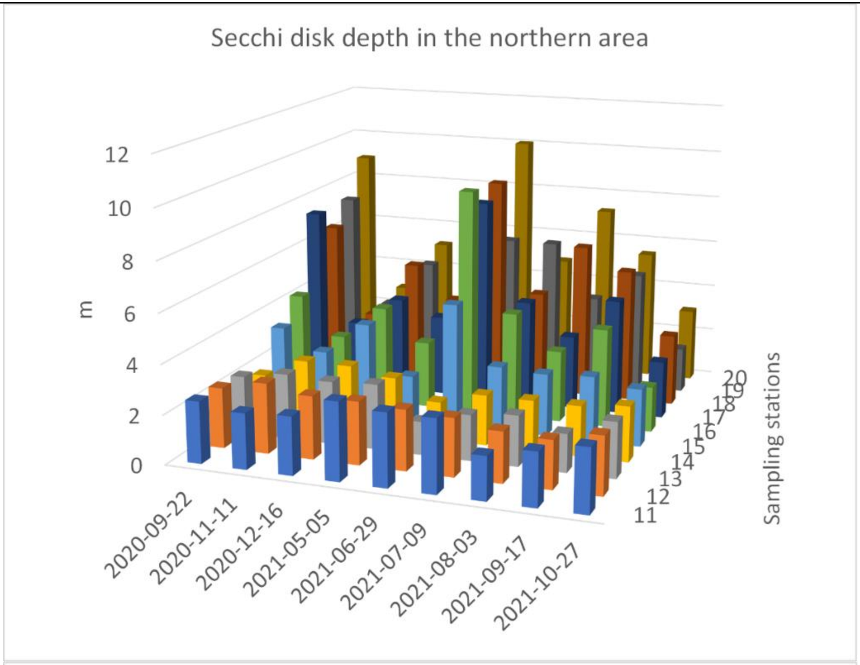
The supplementary materials of this chapter were published in the journal Remote Sensing.

Title: Accurate Estimation of Chlorophyll-a Concentration in the Coastal Areas of the Ebro Delta (NW Mediterranean) Using Sentinel-2 and Its Application in the Selection of Areas for Mussel Aquaculture.

Authors: Margarita Fernández Tejedor, Jorge Enrique Velasco, Eduard Angelats

Reference:

Fernández-Tejedor, M.; Velasco, J.E.; Angelats, E. Accurate Estimation of Chlorophyll-a Concentration in the Coastal Areas of the Ebro Delta (NW Mediterranean) Using Sentinel-2 and Its Application in the Selection of Areas for Mussel Aquaculture. Remote Sens. 2022, 14, 5235. https://doi.org/10.3390/rs14205235



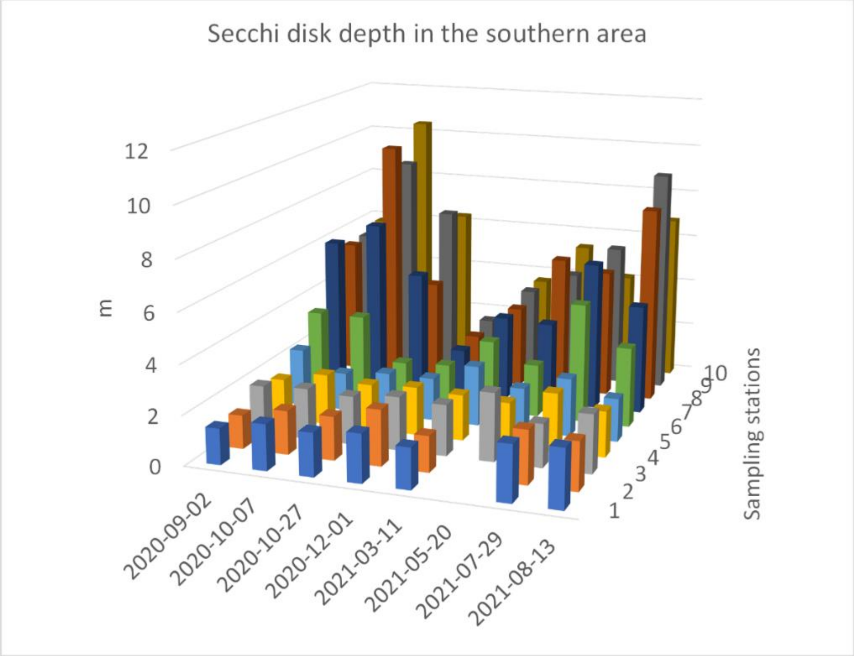
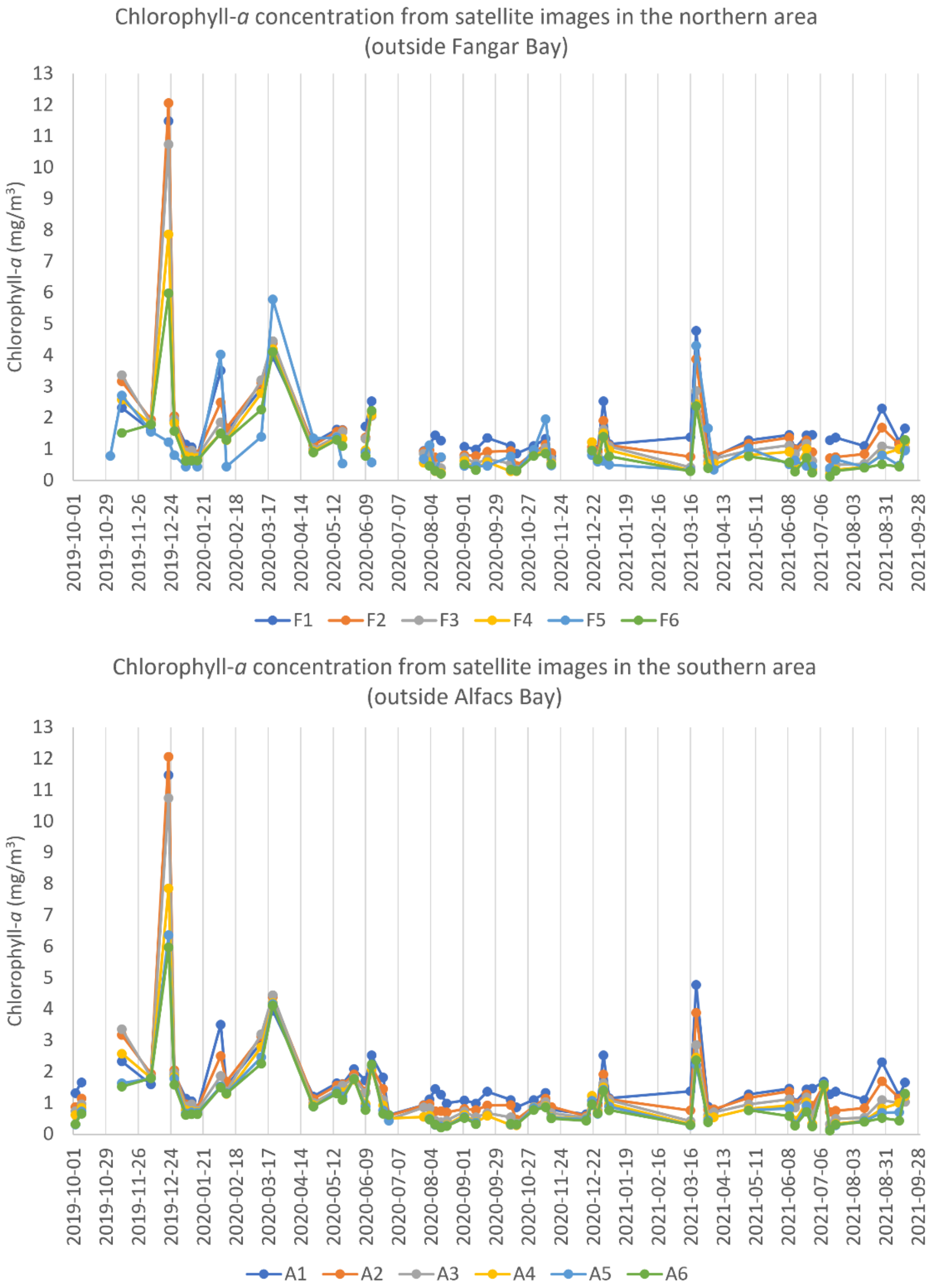


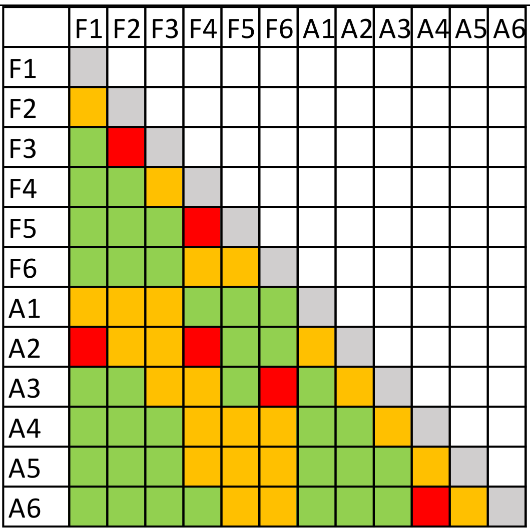
Figure 2S- 1. Secchi depth (m) at the different sampling stations. Note that the axis of sampling stations is reversed in the graphs showing chlorophyll-*a* concentration from Figure 3.



Chlorophyll-*a* concentration from satellite images in the northern area (outside Fangar Bay)

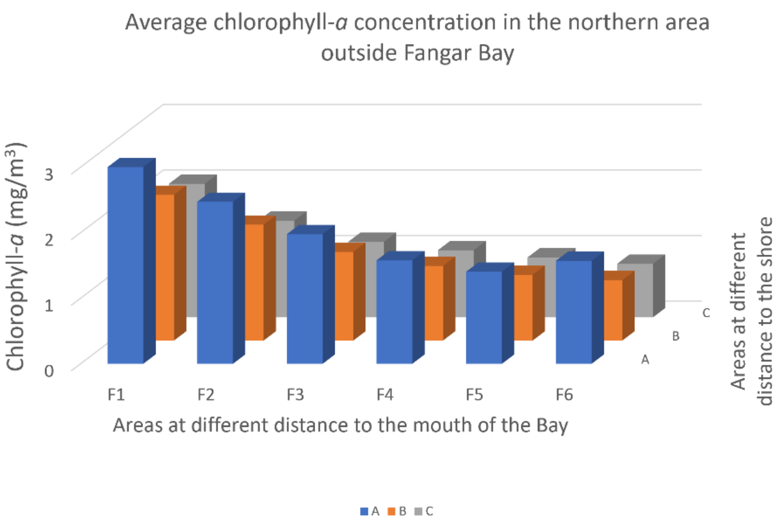
Chlorophyll-*a* concentration from satellite images in the southern area (outside Alfacs Bay)

Figure 2S- 2. Chlorophyll-*a* concentration, from Sentinel-2 images, averaged for each polygon and date outside the embayments.



	F1	F2	F3	F4	F5	F6	A1	A2	A3	A4	A5	A6
F1	▨											
F2	🟧	▨										
F3	🟩	🟥	▨									
F4	🟩	🟩	🟧	▨								
F5	🟩	🟩	🟩	🟥	▨							
F6	🟩	🟩	🟩	🟧	🟧	▨						
A1	🟧	🟧	🟧	🟩	🟩	🟩	▨					
A2	🟥	🟧	🟧	🟥	🟩	🟩	🟧	▨				
A3	🟩	🟩	🟧	🟧	🟩	🟥	🟩	🟧	▨			
A4	🟩	🟩	🟩	🟧	🟧	🟧	🟩	🟩	🟧	▨		
A5	🟩	🟩	🟩	🟧	🟧	🟧	🟩	🟩	🟩	🟧	▨	
A6	🟩	🟩	🟩	🟩	🟧	🟧	🟩	🟩	🟩	🟥	🟧	▨

Figure 2S- 3. Matrix showing the results of the statistical analysis used to detect differences on the chlorophyll-*a* concentration, from Sentinel-2 images, averaged for each polygon and date outside the embayments. The squares colored in green correspond to polygons where statistical significant differences have been found, red color is for polygons where no differences have been detected, orange is for those polygons where no significant differences were found although they may exist.



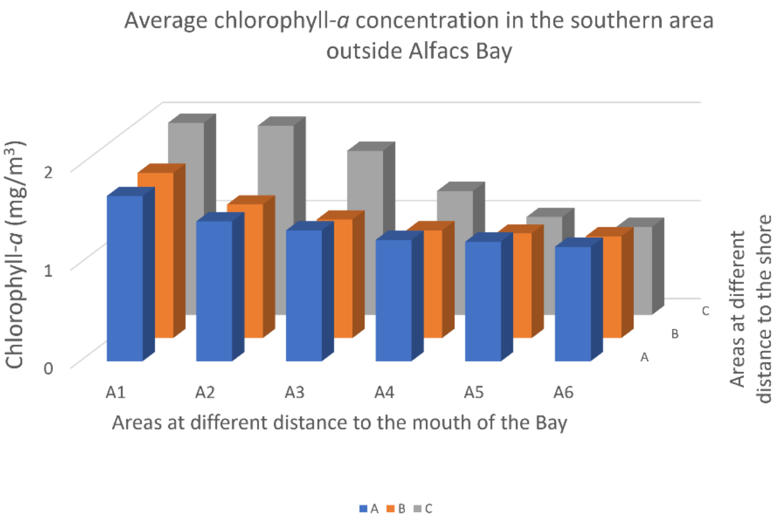


Figure 2S- 4. Chlorophyll-*a* concentration, from Sentinel-2 images, averaged for each of the 3 parts of each polygon outside the embayments, A-C are areas at different distances from the shore inside each polygon.

References

JACUMAR, 2023. Producción de acuicultura. Ministerio de Agricultura, Pesca y Alimentación.

Supporting information of Chapter 3

The supplementary materials of this chapter were published in the journal Harmful Algae.

Title: Toxic marine microalgae and noxious blooms in the Mediterranean Sea: A contribution to the Global HAB Status Report

Authors: Adriana Zingone, Laura Escalera, Katerina Aligizaki, Margarita Fernández-Tejedor, Amany Ismael, Marina Montresor, Patricija Mozetič, Seyfettin Taş, Cecilia Totti.

Reference: Adriana Zingone, Laura Escalera, Katerina Aligizaki, Margarita Fernández-Tejedor, Amany Ismael, Marina Montresor, Patricija Mozetič, Seyfettin Taş, Cecilia Totti, 2021 Toxic marine microalgae and noxious blooms in the Mediterranean Sea: A contribution to the Global HAB Status Report. Harmful Algae, Volume 102, 101843, ISSN 1568-9883, https://doi.org/10.1016/j.hal.2020.101843.

(https://www.sciencedirect.com/science/article/pii/S1568988320301220)

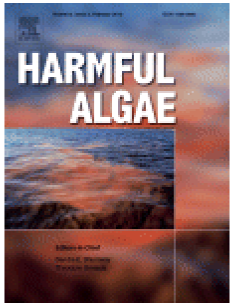


Table 3S- 1. Water discoloration events along the Mediterranean coasts. Species names have been updated following the currently accepted taxonomical nomenclature.

Species	Year	Water colour	Anoxia (Yes/-)	Location	Author	Max. density (cells·L^{-1})	Remarks
Bacillariophyceae							
Chaetoceros socialis	2000	Yellow-brown	-	Alexandria (EG)	Mikhail (2001)	$8.1 \cdot 10^6$	
Chaetoceros sp.	1975	-	-	Marsamxett Harbour (MA)	Fudge (1977)	-	
Hemiaulus sp.	1988	Brown	-	Emilia-Romagna (IT)	Regione Emilia-Romagna (1976-2018)	-	
Lioloma pacificum	2013	Brown-green	-	Emilia-Romagna (IT)	Regione Emilia-Romagna (1976-2018)	-	
Nitzschia, *Hantzschia*	2000		-	Étang du Prévost (FR)	Belin and Soudant (2018)	$1.2 \cdot 10^8$	
Pseudo-nitzschia sp.	2007, 2010	Brown-green	Y	Emilia-Romagna (IT)	Regione Emilia-Romagna (1976-2018)	$6.0 \cdot 10^6$	
Skeletonema cf. *costatum*	2000-2001	Brown	-	Sea of Marmara (TK)	Taş et al. (2009)	$8.0 \cdot 10^6$	
Skeletonema marinoi	2009-2014	Brown	-	Sea of Marmara (TK)	Taş and Yilmaz (2015), Taş (2019)	$5.4 \cdot 10^7$	Events occurred intermittently
Skeletonema marinoi	1978, 1982, 1983-2018	Green	-	Emilia-Romagna (IT)	Regione Emilia-Romagna (1976-2018)	$2.0 \cdot 10^8$	Events occurred intermittently

Skeletonema marinoi, *Eutreptiella pascheri*, *Pseudo-nitzschia* cf. *seriata*	1970	Green and brownish-red	-	Venice Lagoon (IT)	Voltolina (1970)	$4\cdot10^{7}$	
Thalassiosira minima	2013-2014		-	Sea of Marmara (TK)	Taş (2019)	$1.3\cdot10^{7}$	
Mixture of diatoms	1956, 1959	Reddish-brown	-	Nile River mouth (EG)	Halim (1960b)	$2.4\cdot10^{6}$	Total cell density
Chlorophyta							
Micromonas pusilla	2007	Green	-	Miami beach, Alexandria (EG)	Ismael (2014)	$2.1\cdot10^{7}$	
Pyramimonas grossii	2009-2010		-	Sea of Marmara (TK)	Taş and Yilmaz (2015)	$1.6\cdot10^{6}$	
Pyramimonas sp.	2004		-	Campania (IT)	Zingone et al. (2006)	-	
Tetraselmis wettsteinii	1987	Emerald green	-	Gulf of Naples (IT)	Zingone et al. (2006)	$5.0\cdot10^{7}$	
Cryptophyceae							
Plagioselmis prolonga	2009-2014	Green-brown	-	Sea of Marmara (TK)	Taş and Yilmaz (2015), Taş (2019)	$7.8\cdot10^{6}$	Events occurred intermittently
Dictyochophyceae							
Octactis speculum	1983	-	-	SE Gulf of Trieste (SL)	Fanuko (1989)	$6.6\cdot10^{5}$	
*Vicicitus globosus**	2018	Green	-	Marche (IT)	Totti et al. (2019a)	-	
Dinophyceae							
*Alexandrium balechii**	1980-1981	Yellow-brown and oily	-	Campania (IT)	Montresor et al. (1990)	$4\cdot10^{7}$	
Alexandrium insuetum	1991	-	-	Languedoc (FR)	Belin and Soudant (2018)	$1.2\cdot10^{8}$	
Alexandrium insuetum	2003	Brown	-	Amvrakikos Bay (GR)	Nikolaidis et al. (2005)	$2.5\cdot10^{6}$	
Alexandrium mediterraneum	1982	Red tide	-	Emilia-Romagna (IT)	Boni (1983), Boni et al. (1986)	$1\cdot10^{7}$	
*Alexandrium minutum**	1958	-	-	Alexandria (EG)	Halim (1960a)	$2.6\cdot10^{7}$	
*Alexandrium minutum**	2002	-	-	Catalonia (SP)	Garcés et al. (2004)	$4.7\cdot10^{7}$	
*Alexandrium tamarense**	1993	Bright yellow	Y	Veneto (IT)	Sorokin et al. (1996)	$4.0\cdot10^{6}$	

*Alexandrium taylorii**	1997- 2010	Green-yellow	-	Sicily and Sardinia (IT)	Giacobbe and Yang (1999), Penna et al. (2002), Giacobbe et al. (2007), Satta et al. (2010), Satta et al. (2014)	$9.3\cdot x10^6$	Events occurred intermittently
*Alexandrium taylorii**	1994-1998, 2002, 2003	Green	-	Balearic Islands and North Catalonia coast (SP)	Delgado et al. (1997), Garcés et al. (1999b), Basterretxea et al. (2005), Basterretxea et al. (2007)	$9\cdot 10^6$	
Barrufeta bravensis	2012	Green-brown	-	Sardinia (IT)	Satta et al. (2014)	$4.4\cdot 10^6$	
Barrufeta bravensis	2002, 2005	-	-	Catalan coast (SP)	Sampedro et al. (2011)	-	Co-occurring with *A. taylorii*
Cochlodinium sp.	1968	-	-	Juan-les-Pins (FR)	Pincemin (1969)	$8\cdot 10^7$	
Cochlodinium sp.	1972-1975	-	-	Marsamxett Harbour (MA)	Fudge (1977)	-	
Coolia monotis	1982	Brown	-	Veneto (IT)	Tolomio and Cavolo (1985)	-	
Diplopsalis-group	1980-1982	-	-	Emilia-Romagna (IT)	Boni (1983)	$3.2\cdot 10^7$	
Gonyaulax sp.	2004	Red	-	Emilia-Romagna (IT)	Regione Emilia-Romagna (1976-2018)	-	
*Gonyaulax spinifera**	2013	Brown	-	Thermaikos Gulf (GR)	Aligizaki et al. (2016)	$6.4\cdot 10^5$	
*Gonyaulax spinifera**	2007	Red	-	Emilia-Romagna (IT)	Regione Emilia-Romagna (1976-2018)	$2.3\cdot 10^7$	
Gymnodinium impudicum	1988-1992, 1993, 1995-1999	-	-	Valencia and Catalonia (SP)	Fraga et al. (1995), Vila et al. (2001)	$5.9\cdot 10^6$	Blooms are recurrent
Gymnodinium impudicum	1999	Red	-	Lake Tunis (TU)	Turki and El Abed (2001)	$1.7\cdot 10^6$	
Gymnodinium instriatum	2012	Green-brown	-	Sardinia (IT)	Satta et al. (2014)	$7.3\cdot 10^5$	
Gymnodinium litoralis	2009	-	-	Catalonia (SP)	Reñé et al. (2011)	$1.0\cdot 10^6$	Blooms are recurrent
Gymnodinium spp.	1981, 1983, 1984	Green	-	Emilia-Romagna and Gulf of Trieste (IT)	Boni et al. (1986), Fanuko (1989)	$2.0\cdot 10^7$	
Heterocapsa rotundata	1980, 1981, 1998, 2018	Brick-red	-	Emilia-Romagna (IT)	Boni (1983)	$9.1\cdot 10^7$	
Heterocapsa triquetra	2007, 2013,2014	Orange-brownish	-	Sea of Marmara (TK)	Taş (2015), Taş (2019)	$2.1\cdot 10^7$	Events occurred intermittently
*Karlodinium corsicum**	1993	Green	Y	Corse (FR)	Paulmier et al. (1995)	$1.2\cdot 10^9$	

Kryptoperidinium foliaceum	1979	-	-	Emilia-Romagna (IT)	Boni (1983)	$5.0\cdot10^{6}$	
Lepidodinium chlorophorum	1976-1987	Green	-	Emilia-Romagna and northern Adriatic (IT)	Regione Emilia-Romagna (1976-2018), Boni (1983), Artegiani et al. (1985)	$2.3\cdot10^{8}$	Events occurred intermittently
Lepidodinium chlorophorum	2004-2006	-	-	Balearic Islands (SP)	Illoul et al. (2007)	$1.2\cdot10^{7}$	
*Lingulodinium polyedra**	1976-1998	Brown, greenish	-	Emilia-Romagna and Marche (IT)	Regione Emilia-Romagna (1976-2018), Boni (1983), Lalami-Taleb et al. (1985), Ammazzalorso et al. (1990)	$1.3\cdot10^{8}$	Events occurred intermittently
Margalefidinium cf. *polykrikoides**	2018, 2019	Yellow-brownish	-	Apulia (IT)	Roselli et al. (2020)	$2.4\cdot10^{7}$	
Noctiluca scintillans	1971	-	-	Catalan coast (SP)	(Lopez and Arte, 1971)	$3.8\cdot10^{5}$	
Noctiluca scintillans	1976-2004; 2011-2016	Orange, slightly red to brown, gelatinous aspect	-	Gulf of Trieste, Emilia-Romagna, Marche and Veneto (IT)	Regione Emilia-Romagna (1976-2018), Bianchi et al. (1981), Boni (1983), Fonda Umani et al. (2004), Regione Veneto (2004), Regione Marche (2015)	$1.0\cdot10^{8}$	Events occurred intermittently
Noctiluca scintillans	2003	Red	-	Sea of Marmara (TK)	Türkoğlu (2013)	$2.2\cdot10^{5}$	
Noctiluca scintillans	1984	Red	-	Provence (FR)	Laborde et al. (1986)	$3.0\cdot10^{6}$	
Noctiluca scintillans	2000-2004; 2017	Red	-	Thermaikos Gulf (GR)	Nikolaidis et al. (2005), Genitsaris et al. (2019)	$5.4\cdot10^{6}$	3-4 times per year
Peridinium quinquecorne	1999	Red	-	Lake Bizerte (TU)	Turki and El Abed (2001)	$1.4\cdot10^{7}$	
Peridinium quinquecorne	2007	Brown-red	-	Miami beach, Alexandria (EG)	Ismael (2014)	$9.9\cdot10^{6}$	
Plectodinium nucleovolvatum	1968	Reddish-brown	-	Pietà Creek (MA)	Micallef and Bannister (1969)	$4.2\cdot10^{8}$	
Polykrikos sp.	2014	Pink	-	Emilia-Romagna (IT)	Regione Emilia-Romagna (1976-2018)	-	
*Prorocentrum cordatum**	1962, 1977	Yellow-brown	-	Campania and Emilia-Romagna (IT)	Yamazi (1964), Boni (1983)	$2.9\cdot10^{7}$	
*Prorocentrum cordatum**, *P. balticum*	1994	-	-	Étang de Berre (FR)	Belin and Soudant (2018)	$1.7\cdot10^{8}$	
*Prorocentrum cordatum**	2000-2001, 2007	Brown-red	-	Sea of Marmara (TK)	Taş and Okuş (2011), Taş and Yilmaz (2015)	$7.0\cdot10^{7}$	
Prorocentrum micans	1976-1977; 1984,1987	Reddish	-	Emilia-Romagna (IT)	Regione Emilia-Romagna (1976-2018), Boni (1983)	$1.2\cdot10^{7}$	

Prorocentrum micans	2003, 2015	Brown-red and mucilage	-	Sea of Marmara (TK)	Türkoğlu (2008), Ergül et al. (2018)	$1.8\cdot10^{7}$	
Prorocentrum cf. *obtusidens*	2001	Brown	-	Thermaikos Gulf (GR)	Nikolaidis et al. (2005)	$1.2\cdot10^{6}$	
Prorocentrum redfieldii	2000	Brown	-	Thermaikos Gulf (GR)	Nikolaidis et al. (2005)	$6.0\cdot10^{6}$	
Prorocentrum scutellum	1982	-	-	Emilia-Romagna (IT)	Boni (1983)	$3.0\cdot10^{7}$	
Prorocentrum scutellum	1999	Red	-	Sea of Marmara (TK)	Taş and Okuş (2004)	$2.4\cdot10^{6}$	
Prorocentrum triestinum	1992	Yellow-brown	-	Alexandria (EG)	Zaghloul (1995)	$7.5\cdot10^{7}$	
Prorocentrum triestinum	1983-2017	Green, brown	-	Campania, Lazio and Emilia-Romagna (IT)	Regione Emilia-Romagna (1976-2018) Zingone et al. (2006), Bianco et al. (2012)	$1.3\cdot10^{7}$	
Prorocentrum triestinum	1970, 1971	Red-brown	-	Marsamxett Harbour (MA)	Fudge (1977)	$2.7\cdot10^{8}$	
Protoperidinium depressum	1969	Red patches	Y	Emilia Rogmana (IT)	Froglia (1970)	-	
Protoperidinium ovatum	1975	-	-	Banyuls-sur-Mer (FR)	Neveux and Soyer (1976)	-	
Ptychodiscus sp.	1972-1975	-	-	Marsamxett Harbour (MA)	Fudge (1977)	-	
Scrippsiella acuminata	1992, 1999, 2006	Yellow-brown /Brown-red	-	Alexandria (EG)	Mikhail (1997), Ismael and Halim (2000), Moustafa et al. (2009)	$6.1\cdot10^{6}$	
Scrippsiella acuminata	1982, 1992, 2018	-	-	Emilia-Romagna (IT)	Regione Emilia-Romagna (1976-2018), Boni (1983)	$1.1\cdot10^{7}$	
Scrippsiella acuminata	2009-2014	Orange-brown	-	Sea of Marmara (TK)	Taş and Yilmaz (2015), Taş (2019)	$2.3\cdot10^{6}$	
Scrippsiella spp.	1970	Brown	-	Castellón (SP)	San Feliú et al. (1971)	$2.1\cdot10^{7}$	
Euglenophyceae							
Euglena viridis	2009-2010	Green	-	Sea of Marmara (TK)	Taş and Yilmaz (2015)	$1.1\cdot10^{7}$	
Eutreptiella marina	2009-2014	Green	-	Sea of Marmara (TK)	Taş and Yilmaz (2015), Taş (2019)	$3.0\cdot10^{7}$	
Eutreptiella sp.	2001	Green	-	Sea of Marmara (TK)	Taş et al. (2009)	$3.0\cdot10^{6}$	
Haptophyceae							
Holococcolithophora sphaeroidea	2001	White-green-turquoise	-	Tarragona harbour (SP)	Cros et al. (2002)	$1.3\cdot10^{7}$	
Emiliana huxleyi	2003-2004	-	-	Sea of Marmara (TK)	Türkoğlu (2008), Türkoğlu (2010)	$2.5\cdot10^{8}$	

*Prymnesium parvum**	1977-1978	Red-chocolate	-	Catalan coast (SP)	Comín and Ferrer (1978)	$2.0\cdot10^{8}$	
Raphidophyceae							
*Chattonella subsalsa**	1956	Brown	-	Algiers (AL)	Hollande and Enjumet (1957)	$2.0\cdot10^{6}$	
*Chattonella subsalsa**	1987	Brown	-	Campania (IT)	Zingone et al. (2006)	-	
*Chattonella subsalsa**	1963-1966	-	-	Barcelona (SP)	Margalef (1968)	$3.8\cdot10^{7}$	
*Fibrocapsa japonica**	1998-2018	Brick-red	-	Marche and Emilia-Romagna (IT)	Boni et al. (1983), Mattei et al. (2005), Cucchiari et al. (2008), Totti et al. (2019a)	$5.0\cdot10^{7}$	Events occurred intermittently
*Heterosigma akashiwo**	2009-2014	Yellow-brown	-	Sea of Marmara (TK)	Taş and Yilmaz (2015), Dursun (2016), Taş (2019)	$3.1\cdot10^{7}$	Events occurred intermittently
Ciliates							
Mesodinium rubrum	2006	Dark green-brownish	-	Gulf of Naples (IT)	Siano et al. (2006)	$2.0\cdot10^{6}$	
Mesodinium rubrum	2017	-	-	Thermaikos Gulf (GR)	Genitsaris et al. (2019)	$7\cdot10^{5}$	

*This is a toxic species but the discoloration was not associated to any toxicity.

CR: Croatia; EG: Egypt; FR: France; GR: Greece; IT: Italy; MA: Malta; SL: Slovenia; SP: Spain; TU: Tunisia; TK: Turkey.

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Figure 3S- 1. Distribution of *Pseudo-nitzschia* spp., *Nitzschia* spp. and *Halamphora* spp. in the Mediterranean Sea. The 3 genera include both species that produce the toxin Domoic Acid and non-toxic species, and are often identified only at the genus level. Data from OBIS (https://obis.org/).

Supporting information of Chapter 4

The supplementary materials of this chapter were published in the journal Frontiers in Marine Science.

Title: The endoparasite *Perkinsus olseni* affecting the Mediterranean mussels (*Mytilus galloprovincialis*) in the Italian and Spanish waters: A new possible threat for mussel aquaculture and wild animal population.

Authors: Francesca Carella, Margarita Fernandez Tejedor, Grazia Villari, Karl Blyth Andree and Gionata De Vico.

Reference: Carella F, Fernandez Tejedor M, Villari G, Andree KB and De Vico G (2023) The endoparasite Perkinsus olseni affecting the Mediterranean mussels (Mytilus galloprovincialis) in the Italian and Spanish waters: A new possible threat for mussel aquaculture and wild animal population. Front. Mar. Sci. 10:1116837. doi: 10.3389/fmars.2023.1116837

Table 4S- 1. The pairwise genetic distance between isolates

P._qugwadi_P._yessoensis_Canada																					
_P._marinus_C._virginica_USA	0,322																				
P._qugwadi_P._yessoensis_Canada	0,0203	0,3100																			
Perkinsus_sp._P._undulata_Thailand	0,2690	0,0360	0,2588																		
P._marinus_C._virginica_USA	0,3223	0,0090	0,3116	0,0396																	
P._olseni_Pitar_rostrata_Uruguay	0,2663	0,0397	0,2561	0,0053	0,0433																
P._chesapeaki_M._balthica_USA	0,3543	0,1242	0,3473	0,1141	0,1289	0,1143															
P._olseni_R._philippinarum_Italy	0,2987	0,0440	0,2899	0,0018	0,0487	0,0071	0,1287														
P._marinus_C._virginica_USA	0,3046	0,0048	0,2942	0,0406	0,0000	0,0444	0,1318	0,0501													
P._mediterraneus_O.edulis_Balearic_island	0,3090	0,0531	0,2988	0,0541	0,0579	0,0579	0,1284	0,0538	0,0579												
P._chesapeaki_M._arenaria_USA	0,3412	0,1299	0,3322	0,1188	0,1349	0,1190	0,0221	0,1371	0,1349	0,1404											
P._honshuensis_R._philippinarum_Japan	0,3302	0,0917	0,3180	0,0632	0,0948	0,0634	0,1505	0,0646	0,0573	0,0400	0,1424										
P._beihaiiensis_C._hongkongensis_China	0,3317	0,1077	0,3215	0,0991	0,1109	0,1048	0,1592	0,1045	0,1109	0,1171	0,1677	0,1073									
P._olseni_Austrovenus_stutchburyi_New_Zealand	0,2648	0,0406	0,2543	0,0072	0,0443	0,0000	0,1152	0,0090	0,0443	0,0595	0,1188	0,0556	0,1046								
P._olseni_Austrovenus_stutchburyi_New_Zealand	0,2938	0,0484	0,2848	0,0054	0,0532	0,0018	0,1290	0,0063	0,0532	0,0569	0,1353	0,0518	0,1075	0,0054							
P._mediterraneus_O.edulis_Balearic_Islands_Spain	0,3073	0,0498	0,2972	0,0468	0,0547	0,0506	0,1268	0,0475	0,0547	0,0109	0,1419	0,0336	0,1203	0,0523	0,0506						
P._olseni_R._philippinarum_Ebro_Delta_Spain	0,2792	0,0433	0,2692	0,0019	0,0485	0,0076	0,1274	0,0000	0,0485	0,0451	0,1345	0,0469	0,1111	0,0095	0,0069	0,0381					
P_olseni_R._decussatus_France	0,2444	0,0390	0,2394	0,0057	0,0429	0,0115	0,1195	0,0038	0,0444	0,0630	0,1277	0,0763	0,1004	0,0138	0,0118	0,0551	0,0042				
KJ569360.1_P._mediterraneus	0,3056	0,0498	0,2955	0,0486	0,0547	0,0524	0,1252	0,0475	0,0547	0,0093	0,1404	0,0304	0,1171	0,0541	0,0506	0,0047	0,0381	0,0571			
P._olseni_V._aurea_Sardinia_Italy	0,2795	0,0452	0,2700	0,0036	0,0503	0,0089	0,1321	0,0016	0,0508	0,0563	0,1402	0,0588	0,1111	0,0108	0,0083	0,0497	0,0017	0,0058	0,0497		
P._beihaiensis_C._rhizophorae_Brasil	0,3190	0,1098	0,3081	0,1053	0,1132	0,1111	0,1661	0,1147	0,1132	0,1212	0,1735	0,1075	0,0115	0,1109	0,1179	0,1246	0,1164	0,1072	0,1212	0,1153	
P._olseni_R._decussatus_Galicia_Spain	0,2982	0,0439	0,2894	0,0018	0,0486	0,0070	0,1285	0,0000	0,0500	0,0537	0,1369	0,0645	0,1043	0,0090	0,0063	0,0474	0,0000	0,0038	0,0474	0,0016	0,1145

P._chesapeaki_C._columbiensis_Panama	0,3384	0,1201	0,3294	0,1097	0,1250	0,1117	0,0410	0,1290	0,1250	0,1324	0,0249	0,1309	0,1581	0,1115	0,1288	0,1308	0,1257	0,1178	0,1292	0,1318	0,1633	0,1288												
P._chesapeaki_C._rhizophorae_Brazil	0,3384	0,1201	0,3294	0,1097	0,1250	0,1117	0,0410	0,1290	0,1250	0,1324	0,0249	0,1309	0,1581	0,1115	0,1288	0,1308	0,1257	0,1178	0,1292	0,1318	0,1633	0,1288	0,0000											
P._olseni_R._philippinarum_Korea	0,3041	0,0431	0,2971	0,0018	0,0478	0,0070	0,1279	0,0000	0,0500	0,0537	0,1369	0,0742	0,1043	0,0090	0,0063	0,0474	0,0000	0,0038	0,0474	0,0016	0,1145	0,0000	0,1288	0,1288										
P._beihaiensis_M._galloprovincialis_Tokyo	0,3288	0,1038	0,3186	0,0965	0,1071	0,1022	0,1542	0,1023	0,1071	0,1134	0,1629	0,1034	0,0062	0,1020	0,1053	0,1166	0,1087	0,0974	0,1134	0,1071	0,0000	0,1021	0,1548	0,1548	0,1021									
P._beihaiensis_M.galloprovincialis_Tokyo	0,2356	0,0707	0,2289	0,0635	0,0759	0,0687	0,1061	0,0662	0,0759	0,0957	0,1111	0,0785	0,0172	0,0685	0,0685	0,0932	0,0658	0,0643	0,0932	0,0660	0,0102	0,0660	0,0985	0,0985	0,0660	0,0074								
P._beihaiensis_M._galloprovincialis_Tokyo	0,2356	0,0755	0,2289	0,0682	0,0807	0,0734	0,1106	0,0709	0,0807	0,1003	0,1156	0,0833	0,0269	0,0732	0,0732	0,0977	0,0708	0,0691	0,0977	0,0682	0,0202	0,0707	0,1030	0,1030	0,0707	0,0123	0,0025							
P._olseni_Anadara_trapezia_Australia	0,2988	0,0468	0,2898	0,0018	0,0516	0,0036	0,1323	0,0047	0,0516	0,0553	0,1385	0,0534	0,1059	0,0054	0,0079	0,0490	0,0052	0,0079	0,0490	0,0066	0,1162	0,0047	0,1304	0,1304	0,0047	0,1037	0,0660	0,0707						
P._olseni_P._malabarica_India	0,2989	0,0492	0,2900	0,0071	0,0540	0,0124	0,1349	0,0078	0,0484	0,0521	0,1353	0,0494	0,1027	0,0072	0,0047	0,0458	0,0017	0,0135	0,0458	0,0082	0,1128	0,0078	0,1272	0,1272	0,0078	0,1005	0,0635	0,0682	0,0032					
P._olseni_M_galloprovincialis_Campania_(Italy)	0,3172	0,1115	0,3069	0,0404	0,1162	0,0335	0,1900	0,0705	0,0954	0,0997	0,1721	0,1165	0,1585	0,0234	0,0564	0,0949	0,0458	0,0438	0,0965	0,0566	0,1594	0,0704	0,1656	0,1656	0,0815	0,1568	0,0763	0,0810	0,0580	0,0666				
P._olseni_M_galloprovincialis_Campania_(Italy)(2)	0,3172	0,1115	0,3069	0,0404	0,1162	0,0335	0,1900	0,0705	0,0954	0,0997	0,1721	0,1165	0,1585	0,0234	0,0564	0,0949	0,0458	0,0438	0,0965	0,0566	0,1594	0,0704	0,1656	0,1656	0,0815	0,1568	0,0763	0,0810	0,0580	0,0666	0,0000			
P.olseni M_galloprovincialis Delta ebro 1	0,2998	0,0455	0,2911	0,0070	0,0502	0,0000	0,1270	0,0077	0,0516	0,0569	0,1353	0,0645	0,1075	0,0000	0,0047	0,0506	0,0087	0,0133	0,0506	0,0098	0,1179	0,0077	0,1288	0,1288	0,0077	0,1053	0,0685	0,0732	0,0063	0,0124	0,0738	0,0738		
P.olseniM_galloprovincialis Delta ebro 2	0,2760	0,0412	0,2657	0,0078	0,0466	0,0000	0,1225	0,0090	0,0466	0,0466	0,1280	0,0484	0,1095	0,0000	0,0054	0,0412	0,0089	0,0150	0,0412	0,0107	0,1150	0,0089	0,1207	0,1207	0,0089	0,1070	0,0625	0,0678	0,0072	0,0072	0,0328	0,0328	0,0000	
P._olseni_H._laevigata_Australia	0,3057	0,0416	0,2987	0,0000	0,0462	0,0053	0,1263	0,0015	0,0484	0,0521	0,1353	0,0726	0,1027	0,0072	0,0047	0,0458	0,0017	0,0057	0,0458	0,0033	0,1128	0,0015	0,1272	0,1272	0,0015	0,1005	0,0635	0,0682	0,0032	0,0062	0,0800	0,0800	0,0061	0,0072