DTU Aqua
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Colophon

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Cover photo: Exposed Pacific oysters (*Magallana gigas*) in Klosterbugten, Limfjorden 2018. Photo: Pedro S. Freitas

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Preface

This report is based on two research projects (“Udvikling af strategier for bekæmpelse af stillehavsøsters i skaldyrvande j.nr. 33113-I-17-094” and ”Nye metoder til sortering og forarbejdning af stillehavsøsters j.nr. 33112-I-17-044”) that have received financial support from the European Maritime and Fisheries Fund and the Danish Fisheries Agency. Based on discussions and inputs from multiple stakeholders e.g., fishers, managers, and processing industries, DTU Aqua subsequently and independently of others selected the topics included in the two projects and the results from the two projects are reported in this common report.

Each chapter reflects the views only of the authors listed for a specific chapter. Authors cannot be held responsible for any views or use which may be made of the information contained in other chapters. The summary and recommendations are written by DTU Aqua.

Kgs. Lyngby, December 2022

Pedro S. Freitas & Pernille Nielsen

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Summary

Background
This report is the final delivery of the two projects: Development of strategies for control of the Pacific oyster in Danish waters (Udvikling af strategier for bekæmpelse af stillehavsøsters i skaldyrvande) and Development of sorting and processing methods for Pacific oysters (Nye metoder til sortering og forarbejdning af stillehavsøsters). The projects were initiated based on a stakeholder feedback workshop hosted by DTU Aqua in 2017 and financed by grants from European Maritime and Fisheries Fund and the Danish Foreign Ministry development program for marine innovation.

Aim
The main purpose of the projects was to develop methods to mitigate the spread of the invasive Pacific oyster in Danish coastal areas with current or future possibilities for bivalve fisheries. The focus was to develop methods primarily based on initiatives run by commercial fisheries to initiate common and sustainable management strategies for utilisation of the invasive Pacific oyster.

Summary of results
Short summaries of the results for each of the individual chapters are listed in the section below.

Monitoring and distribution of Pacific oysters in selected areas
The successful establishment of wild and naturally spreading populations of the invasive Pacific oyster (*Crassostrea gigas*, syn. *Magallana gigas*) in northern Europe in recent decades, together with its recent expansion in Danish coastal waters, where it has become widely distributed, raises concerns from both ecological and economical points of view. The Pacific oyster is capable of causing significant changes to coastal ecosystems and potentially negatively impact native bivalve species from competition for resources e.g. food and habitat, or from co-travelling predators, pathogens and parasites. This could affect the Danish shellfish fishery for blue mussels, native flat oysters and cockles that represents 94% of the shellfish production in Denmark.

The status and evolution of Pacific oyster (*C. gigas*) populations in Denmark were assessed in the Limfjorden, the largest Danish fjord, and also the Isefjord, owing to a multitude of factors including: (i) the recent expansion of Pacific oyster in Scandinavia in the last 10-15 years; (ii) the perceived low-medium bioinvasion risk of Pacific oyster in sublittoral habitats; (iii) the recurrent anecdotal reports of increased Pacific oyster populations in inner Danish coastal waters and (iv) the potential threat to native bivalve populations and coastal habitats.

In 2019, Pacific oysters were not found in deep areas in the Isefjord, but only in a few shallow areas at very low densities (<0.003 oysters/m^2), which was one to two orders of magnitude lower than in 2007. Although continuously present in the Isefjord since its introduction approximately four decades ago, Pacific oyster population has not expanded and remains at low densities, suggesting that some habitat and/or environmental factors are limiting its successful expansion.

Contrary to the Isefjord, Pacific oysters were found in significant abundance, up to 2 oysters/m^2 in deeper areas and over 500 oysters/m^2 in shallow areas, respectively, of the western and central Limfjorden. In deep areas, Pacific oysters were most abundant in European flat oyster fishing grounds, while in shallow areas Pacific oysters were widespread, often forming biogenic reefs in littoral and sublittoral habitats. Pacific oyster biomass in the Limfjorden in 2019 was conservatively estimated to

approximately 10,000 tons, with shallow areas accounting for 71.4%, of which 22.6% in dense reef populations, while deep areas accounted for 28.6%.

Pacific oyster at two and three years of age dominated, but individuals reached sizes corresponding to significantly more than five years of age, on occasions with marked differences between sites. Evidence of frequent, if not regular, and recent recruitment was observed, even after significant mortality of ca. 60% due to the severe winter of 2018.

A clear increase in abundance was observed in the last two decades, with density increasing by one to four orders of magnitude from <0.01-3 oysters/m^2 in 2006 and 2011 to several 10's or even 100's of oysters/m^2 in 2019 and 2020.

The Pacific oyster is well established in the Limfjorden, with its population following a similar trajectory and phases of invasion as elsewhere in Northern Europe and is currently in the final adjustment phase undergoing significant fluctuations. The lower abundance and slower timing of population evolution in the Limfjorden relative to the Wadden Sea are likely due to differences in habitat, environmental and biological factors affecting growth rate, recruitment, and mortality. Taken together with the absence of expansion in the Isefjord, population evolution of Pacific oysters after introduction is clearly determined not only by biological factors but also strongly by local habitat and environmental factors.

In the Limfjorden, the greatest expansion of Pacific oysters occurred not only in littoral biogenic reefs, but mainly in sublittoral sediments and sublittoral biogenic reef habitats. These results contrast with previous suggestions that Pacific oysters in Scandinavia only pose low to moderate expansion and habitat impact risks in these two habitats. The ecosystem impacts of the large populations of Pacific oysters observed in the Limfjorden are unknown and warrant further studies.

Testing different aerial drone mounted sensors and developing image analysis-based algorithms to optimise mapping of Pacific oysters in coastal areas

Aerial drones can be useful for mapping marine benthic habitats in shallow areas. However, Pacific oysters are often white/light-coloured and might therefore be difficult to separate from other light-coloured hard substrate e.g., stones and shells or light-coloured soft sediments. Within this project, aerial drones mounted with different sensors (e.g., RGB-camera, multispectral sensor, or LiDAR sensors) to map Pacific oysters in either shallow waters in the Limfjorden or in tidal areas at low tide in the Wadden Sea, have been tested. All three sensors successfully cover relatively larger areas in terms of mapping the extent of the reefs and individual oysters. However, it becomes more time consuming and difficult to quantify the total biomass or assess the coverage of the oysters as the post processing of the RGB-camera and multispectral sensor images were challenged by site specific and environmental conditions. Further optimisation is needed to potentially be able to develop a successful segmentation of the oysters by using supervised learning methods.

Use of the LiDAR sensor to map the 3D structure of a mixed blue mussel and Pacific oyster bed provided detailed information about the height dynamics of the bed. Application of different interpolation methods to estimate the biomass of blue mussels and Pacific oysters showed similar results and no significant correlation was observed between biomass (kg m^{-2}) and the height of the bed, which could be due to relative low numbers of ground truth samples. A high intensity ground truth sampling of the mapped mixed mussel-Pacific oyster bed is recommended to examine if the height of the mixed bed correlates with the biomass (kg m^{-2}) or size of either blue mussels or Pacific oysters.

Genetic characterization of the Pacific oyster population in Danish waters
The Pacific oyster was introduced in Denmark via aquaculture in the 1970s and 1980s and now inhabits a large part of the Danish coasts including Limfjorden and Isefjord. Little is known about the current genetic status and characteristics of this invasive species in Danish waters, which is crucial for generating knowledge about their past and future spread. We have undertaken a study to analyse the genetic status of the population of Pacific oysters in Danish waters. The aims were to investigate: i) the presence of genetically separated stocks (populations), within the North Sea/Baltic Sea region in general, and within the Limfjorden area in particular ii) the genetic relationships among the different stocks, the migration (gene flow) between them and their (genetically effective) population sizes and iii) the potential of genetically based local adaptation of the stocks found in Danish waters. We first developed a panel of Single-Nucleotide Polymorphism (SNP) DNA markers that can provide information about separate stocks and provide an indication of whether the stocks possess specific environmental adaptations, using previously generated knowledge about the genome of the Pacific oyster. We used this SNP-panel, with 96 SNPs, to analyse ~1200 individuals collected as part of the population survey (see above) from different areas in Danish waters, together with three foreign stocks (the Netherlands, Sweden and Norway).

Our results show that the Pacific oysters in the Limfjorden and Isefjord represent two separate stocks. We observed the presence of very high levels of gene flow/migration between oysters sampled at different geographical localities within the Limfjorden, as well as localities in Sweden and Norway, which also corroborates expected patterns from agent-based dispersal models. Regarding the level of recruitment, the Pacific oysters within Danish waters present relatively large effective population sizes, with no observed relatedness among individuals of different size class and hence recruitment within the locations analysed in this study, which strongly indicate that the number of migrants/colonizers across the Limfjorden include many families and likely from many localities. Finally, our results do not show evidence of local adaptation of the Pacific oysters in Danish waters, however a larger proportion of the oyster genome should be investigated to elucidate whether there are genomic regions under local selection. Overall, the genetic characterization of Pacific oyster populations in Danish waters, and the understanding of the establishment history and current migration among localities was obtained.

Size-age relations in shallow Pacific oysters from the microtidal Limfjorden
This field experiment produced a new assessment of growth and size at age for Pacific oysters in the Limfjorden. A significant relationship was established between total shell and umbo lengths that can be used to estimate total length of dead and damaged shells from umbo measurements or be applied to measurements of growth lines in the umbo to reconstruct shell size and growth of individual oysters at different ages.

Marking of Pacific oyster shells with Calcein to constrain growth during a field growth experiment, and thus constrain ageing and geochemical analysis of the last annual growth increment, proved unsuccessful. The likely explanation is the lack of incorporation of Calcein in the shell, even though new shell growth was observed at the time of marking.

The determination of Pacific oyster age using growth lines/marks in acetate peels of the umbo of the flat valve by use of annual cyclic variations in shell magnesium (Mg) and Strontium (Sr) composition to age Pacific oysters from the Limfjorden proved unreliable. Shell Mg and Sr concentration and annual cycles showed significant inter- and intra-shell variation, which rendered ages obtained from shell Mg and Sr annual cycles unreliable when compared with umbo growth line ages. It is possible that highly variable environmental conditions in the very shallow areas of the Limfjorden significantly

affect the Mg and Sr composition of Pacific oyster shells, disturbing preservation of annual cycles. Since the Pacific oysters used in this study came from very shallow locations (0.5 to 1.0 m depth), oysters experienced different environmental conditions during growth due to small changes in depth. In the Limfjorden, tidal amplitude is only 5-20 cm and water level vary in a non-cyclical way determined by short-term meteorological forcing, which can lead to strong changes in temperature, salinity, or emersion.

The strength of the size-age relationship obtained by fitting the von Bertalanffy growth function to the length-at-age was not strong compared to other studies on Pacific oysters. A possible explanation is the large phenotypical and morphological plasticity of Pacific oysters where shells can vary in shape and growth depending on population structure (e.g. in reefs or dense populations, in clumps or individually) and environmental conditions (e.g. food supply, depth, wave exposure, bottom substrate). Shell width provided a better fit length-at-age than shell length (longest axis), possibly because of the three linear measurements of shell/body size, it is the one that best reflects shell volume and thus may be less variable with growth and environmental conditions than shell length. Growth rates were low relative to other studies in inter-tidal conditions, possibly reflecting harsher conditions of such shallow habitats. However, overall growth performance, which reflects growth rate and maximum length, was similar or higher than in inter-tidal areas of the North Sea.

Pathogen and disease screening of Pacific oysters from Danish waters
Pacific oysters from several water zones in Denmark were screened for pathogens, both from low tidal zones and from areas with higher water depths. The samplings covered several years and were taken at different times of the year, as water temperature often is an important factor for pathogen availability and thereby potential disease outbreaks. The pathogens in question were the virus Oyster Herpes Virus (OsHV-1) and the bacterium *Vibrio aestuarianus*, both known to be important diseases of Pacific oysters. Other targeted pathogens were the parasites *Bonamia ostreae* and *Marteilia refringens*, both being notifiable pathogens according to EU regulation and causing disease in flat oysters as well as the latter pathogen in blue mussels. *Bonamia ostreae* has been found in native European flat oysters (*Ostrea edulis*) in Limfjorden since 2014.

Overall, the molecular disease screening of Pacific oysters in the Limfjorden, the Wadden Sea, and the Isefjord found very low levels of pathogens present. Most of the oysters were found to be without any of the four pathogens chosen for the screening. In general, no pathogens were found in oysters collected in colder months of the year, which correlates with most disease and mortality being observed in Pacific oysters in Europe during warmer summer months. None of the screened oysters were found to be positive for *Bonamia* sp. or *M. refringens*.

Only 28 oysters were collected in the shallow areas of the Isefjord in November 2019. None of these 28 oysters was found to carry any of the four chosen pathogens. This could possibly be due to the low water temperatures normally found in November in shallow waters of the Isefjord, as this is known to be unfavourable for the tested pathogens. However, the low number of oysters are not enough to conclude on the overall health status of Pacific oysters in the Isefjord.

Only two individuals were found positive of the OsHV-1 virus of all Pacific oysters screened, and one of these samples was within the weakly positive range, indicating a low infection level. The two infected oysters were of a total of 134 (1.5 %) oysters collected in Ho Bugt in the Wadden Sea in May 2018. Unfortunately, no oysters were collected from the same area later in the season to check for a possible progression of the pathogen (potentially leading to disease and mortality) during the summer months. However, the results show that OsHV-1 is present in Ho Bugt and potentially other places in

the Wadden Sea, and they indicate that the virus could become an issue for Pacific oysters in the area if mean sea temperatures continue to rise and sudden temperature changes continue to be observed more often.

Four of the eleven batches of Pacific oysters from the Limfjorden screened had individuals testing positive for the bacterium *V. aestuarianus*, whereas *V. aestuarianus* was not observed in the five batches from the Wadden Sea or the one from the Isefjord. Disease caused by *V. aestuarianus* is often seen during warm summers, meaning that this might also become an issue in the Limfjorden in the future in connection with rise in average sea water temperatures.

Experiences and identified challenges in developing sustainable fisheries of Pacific oysters in Danish coastal waters

Analysis of landings of Pacific oysters either by hand collection or as bycatches in the blue mussel and flat oyster fisheries were carried out and supplemented with interviews of different stakeholders to identify possibilities or barriers for further development of a fishery for the Pacific oyster. The factors identified as the main challenges for the further development of a fishery for the Pacific oyster in Denmark were: i) Hand collection requires storage in depots, either as sea-based or land-based. The general perception of the fishers was that there is an inertia with the authorities to engage in a constructive dialog to solve the problems related to the depots; ii) Hand collection is hard work, and upscaling will therefore require mechanical fishery and most likely new tools need to be developed but also new markets; iii) Use of non-selective fishery tools would increase the need for utilisation of larger individuals and clumps, which require development of new processing methods, products and export markets and iv) Fisheries in the deeper areas (>3 m) in the Limfjorden is currently not of interest for the fishers due to larger individuals/clumps and the populations in deeper areas are mainly located within Natura 2000 sites, where fishers prioritise allocation of “area impact” to the profitable blue mussel and flat oyster fisheries.

Developing, testing and environmental assessment of different new mitigation tools

Within this project, three different mitigation tools have been tested; i) mini-dredge for fisheries in shallow areas of individuals/smaller clumps of Pacific oysters (developed and tested), ii) a floating excavator to remove larger clumps/early established reefs (tested) and iii) a sorting equipment to sort mixed catches of blue mussels and Pacific oysters (developed and tested).

The new mini-dredge tool was found to be efficient in capture of the target species. However, it had a significant impact on the benthic community associated with the Pacific oysters. Such impact can be partially mitigated by the return of the by-catch to the local habitat, but not for macroalgae and eelgrass if fishing in these areas. Accordingly, all fishing activities must ensure areas containing eelgrass are avoided.

The floating excavator was highly efficient (100-91%) in the removal of Pacific oysters, at low, medium, and high densities. However, the long lasting impacts on both macroalgae and macrofauna communities were observed 22 months after removal activities. Furthermore, several of the impact plots were still visible by eye or by a change in bathymetry 22 months after removal activities and most of the marks left at the bottom were still visible on drone images.

The sorting equipment was very successful in separating the two species and with low percentage of damaged/broken blue mussels and oysters. The bycatch of blue mussels was relatively high (19%) in the Pacific oyster fraction, as often smaller blue mussels were attached to the oysters. The low bycatch (2.3%) of smaller pacific oysters in the blue mussel fraction provide the possibility for relay of a blue mussel bed almost free of Pacific oysters. Mean bycatch percentages of other species than blue

mussel and pacific oyster were generally <1%, except for the seaweed toothed wrack, which due to the attachment to the bivalves had an average bycatch of 12.3%.

Testing High Pressure Processing (HPP) technology and opening non-commercial sized Pacific oysters and pilot studies of potential new products
Different sized individuals (three categories) and clumps (three categories) of Pacific oysters were collected in the Limfjorden and sent to Ireland, where HPP Tolling used high pressure processing (HPP) to open the Pacific oysters. Opening of all six categories of either large sized individuals or clumps of Pacific oysters using the HPP technology was successful at 400 MPa and with a hold time of one minute. At 400 MPa, oysters were nearly all open, had little shell damage and the meat was slightly whiter and plumped up. Evaluation of the frozen and thawed HPP processed oyster meat indicated a change in texture and it is recommended that the oyster meat is used in products with further processing, e.g., cooking or marinating. The cost for the HPP process was estimated to approximately 4 DKK per kg of alive oysters, which is assessed by the industry to be within a reasonable level in relation to a further development of products based on meat from large and/or clumps of Pacific oysters. However, further investigations and development of specific products are needed to make a conclusion of the feasibility.

General recommendations
Based on the conclusions of the project, the following recommendations related to the development of a sustainable Pacific oyster fishery to mitigate the effects of this invasive marine species in the Danish coastal waters have been formulated:

- The earlier during the establishment of Pacific oysters in an area, the less destructive mitigation measures are needed to remove Pacific oysters. The more complex the population structure gets (from smaller individuals at low densities to higher densities and clumps ending with high-density reefs), the more destructive tools are needed, and the Pacific oysters are of less or no commercial value. To be able to initiate mitigation measures early, systematic monitoring efforts are required both in relation to population development (e.g., biomass, recruitment patterns, densities and genetics), but also in relation to spreading of bivalve pathogens to determine if the spread takes place in and to other areas with other important commercial bivalve species both in relation to protection of commercial fishery interests and in relation to nature conservation management.

- Site-specific conditions e.g., water depth and population structure both between and within areas with Pacific oysters all determine the type of mitigation tool which can be used and thereby control the environmental impact of the mitigation action. Within the project three different mitigation tools were tested. The mini-dredge is recommended in shallow areas with low densities of individuals and smaller clumps, whereas the floating excavator are recommended in shallow areas with higher densities and clumps and could potentially also be used in reef areas. The sorting equipment can be used on-board larger vessel to sort mixed catches of blue mussels and Pacific oysters. Whether the sorting equipment can sort mixed catches of Pacific oysters and flat oysters have not been tested. The sorting equipment could potentially also be used on land to sort mixed catches from the mini-dredge e.g., at the landing sites.

- Implementation of fisheries in shallow areas (<3 m) will need to comply with the relevant legislations. Licences for fisheries with the mini-dredge in shallow areas will overlap with the current depth limit for eelgrass protection in Danish coastal areas and the fishery can therefore

not be regulated by the general depth limit of 3 m for bivalve fisheries in the Limfjorden[1]. It is therefore recommended that the eelgrass is protected by site-specific “eelgrass boxes”, where fishing is not allowed. However, the general buffer zone of 100 m around eelgrass beds (based on a wire length of <100 m in deeper areas) will likely eliminate most areas with Pacific oysters that can be fished with the mini-dredge. During the scientific fishing carried out by DTU Aqua, the wire length was typically <15 m, which indicates that the general buffer zone of 100 m could be reduced. It is therefore recommended that the general buffer zone of 100 m is adjusted to reflect the specific conditions in the shallow areas, where the wire length of the mini-dredge is shorter and Pacific oysters can be located in shallower areas (closer to shore) than the eelgrass beds.

- According to the current licences for fishery of blue mussels or flat oysters, discard of invasive species is not allowed and therefore all caught oysters must be landed. The Pacific oysters of non-commercial interest (large individuals and clumps) are therefore often considered a waste problem by the fishers and traders. Development of cost-efficient processing methods of Pacific oysters, new products and new export markets are required to be able to utilise the large individuals and clumps of Pacific oysters but potentially also utilisation of the caught shells. By-catch is especially a challenge for the fishery with the mini-dredge that will need to be carried out by smaller boats (<10 m), which have limited space onboard the vessel for sorting of the catches. A better commercial utilisation of the total catch (incl. large individuals, clumps, and shells) would potentially improve the cost-efficiency of the mini-dredge fishery.

- Increasing local awareness in the municipalities where the Pacific oysters can influence recreational activities could potentially be an effective and low-cost mitigation action. Stakeholder initiatives can be initiated by e.g., informing in local medias or with stands at local shellfish festivals, teaching high-school and school classes or encourage local municipalities, environmental NGOs and environmental authorities to the engage local stakeholders operating in the shallow areas (e.g., fishers, anglers, spear fishers and kayak clubs) in coordinated actions to clean selected areas from Pacific oysters.

[1] BEK nr 2298 af 03/12/2021

1. Introduction

Within the last five to ten years, populations of Pacific oysters have increased dramatically in several important bivalve fishing areas in Denmark but also within Natura 2000 sites. The fishery organisations consider Pacific oysters (*Magallana gigas*, syn. *Crassostrea gigas*) to be a threat to their primary fisheries of blue mussels (*Mytilus edulis*), European flat oysters (*Ostrea edulis*) and common cockles (*Cerastoderma edule*). The fishery authorities, furthermore, consider Pacific oyster both as a potential risk for protected species and habitats within Natura 2000 sites and to the overall ecological status and the natural biodiversity of the marine ecosystem.

Before the start of the two EMFF-projects, several licences for short-term experimental test-fisheries of Pacific oysters were initiated in different areas, which demonstrated that knowledge of population dynamics, mapping of stocks, proper fishing tools and processing methods as well as assessment of environmental impact by initiating the fisheries were lacking. To provide sufficient documentation and development of the fisheries require both research and development, which was not possible for the fishery organisations and industry to solve by themselves. Consequently, a stakeholder workshop was initiated by DTU Aqua in 2017 and based on the feedbacks, two research projects were developed with the overall aim to develop methods to mitigate the spread of the invasive Pacific oyster in areas in Denmark with current or future possibilities for bivalve fisheries. The focus was to develop methods primarily based on initiatives run by commercial fisheries, as establishing publicly funded programs aimed at mitigating the spread and effects of the invasive Pacific oyster, was envisaged not possible by the different stakeholders.

The two projects were divided into logically coherent topics to be able to effectively assess the development and spread of Pacific oysters, but with a special focus in relation to developing sustainable fishery-based mitigation strategies of Pacific oysters. The two projects included collection of basic information about population densities, population structures, the development and spread of Pacific oysters in Danish waters including their genetic relationship but also screening of bi-valve pathogens. Furthermore, development of site-specific fishing tools and assessment of the environmental impacts, and testing of processing methods of Pacific oysters not suitable (e.g., large individuals and clumps) for the fresh market were also included.

2. Monitoring and distribution of Pacific oysters in selected areas

Authors: Pedro S Freitas, Pernille Nielsen, Antonio Agüera Garcia, Patrick Joyce and Camille Saurel

Typically, invasions of a species into a novel area occur through multiple phases. Firstly, the species must be transported to the new area. Of the many species that are transported to novel areas, very few survive to persist, thus establishment is the next key step. From this point, the new population, if successful, will go through an expansion phase whereby the population increases significantly. After the population has expanded, several processes such as predation, competition, disease infection, environmental conditions and carrying capacity of the habitat may control the population, which enters an adjustment phase to its new environment. The success of the invader in question, and the magnitude and timescale at which these processes occur are likely to vary greatly depending on local conditions.

The majority of Pacific oyster invasions have been documented in intertidal areas of estuaries, small bays, and narrow sounds. Intertidal habitat types that have been invaded include hard substrates, sand- and mudflats, as well as biogenic reefs (e.g., mussel beds). Although sublittoral Pacific oysters are regularly found, due to the greater perceived threat to intertidal ecosystems (Mortensen et al., 2017), investigations into sublittoral population expansion and impacts are rare. In Scandinavia, littoral biogenic reefs have been suggested to be at the greatest risk of invasion and the largest ecosystem effects by Pacific oyster, while sub-littoral habitats were assessed to have low to moderate risk of invasion and moderate ecosystem effects by Pacific oysters (Mortensen et al., 2017).

In Denmark, Pacific oyster was first introduced into the Limfjorden in ~1972 for aquaculture purposes and then subsequently introduced to the Danish Wadden Sea, Horsens Fjord, Lillebælt and Isefjord over the following two decades (Kristensen, 1989; Jensen & Knudsen, 2005). Feral Pacific oyster populations have been documented in Denmark since the 1990s, more recently appearing in reefs, often mixed with blue mussels, in the western Limfjorden and Danish Wadden Sea (Wang et al., 2007; Wrange et al., 2010; Groslier et al., 2014; Holm et al., 2015, 2016).

Pacific oyster cultivation previously took place in multiple areas of Denmark including the Limfjorden and Isefjord (Kristensen, 1989; Jensen & Knudsen 2005; Kristensen and Hoffman, 2006), which are characterised by their micro-tidal nature, shallow depth, large seasonal temperature, and salinity variations. Pacific oyster production ended in 1999 (Nehring, 2011), but cultivation trials happened as recently as 2016 and 2019 (Aquamind, 2016, P. Freitas, personal observation). The few surveys of feral Pacific oyster populations in shallow areas within the Limfjorden showed that regular recruitment occurred in the western Limfjorden at the Agger Tange intertidal reef (Wrange et al., 2010; Groslier et al., 2014; Holm et al., 2015, 2016), but that populations throughout the rest of the Limfjorden were small. Expansion of these populations was suggested to be limited by environmental conditions or the need of more time for the populations to expand (Wrange et al., 2010; Groslier et al., 2014).

The status and evolution of Pacific oyster populations in Denmark were assessed in the Limfjorden, the largest Danish fjord, and the Isefjord owing to a multitude of factors including: i) the recent expansion of Pacific oyster in Scandinavia in the last 10-15 years; ii) the perceived low-medium bioinvasion risk of Pacific oyster in sublittoral habitats; iii) the recurrent anecdotal reports of increased Pacific oyster populations in Danish coastal inner waters and iv) the potential threat to native bivalve populations and coastal habitats.

In this study, surveys were conducted throughout the western Limfjorden to determine: i) the impact of the severe winter of 2018 and the potential previous extent of the Pacific oyster population and ii) the population distribution, structure, and size of Pacific oyster in shallow subtidal (<1 m depth) and deeper areas (>3 m depth) to assess its invasion and expansion status. Similarly, the deep and shallow areas of the Isefjord were surveyed with the same objectives.

2.1 Distribution and population dynamics of Pacific oysters in the Limfjorden

The Limfjorden in northern Jutland is the largest fjord in Denmark, ca. 180 km long with 1000 km of coastline and an area of ca. 1500 km^2 (Figure 2.1.1). The Limfjorden connects in the west to the North Sea through a narrow inlet, while in the east a long and narrow channel connects to the Kattegat. It is a highly eutrophic system made up of several shallow basins averaging ~4.5 m depth, often connected by narrow channels. Saline North Sea water and freshwater inputs are of similar magnitude and a residual eastward flow towards the Kattegat. The Limfjorden is a micro-tidal system (tidal amplitude <30 cm) and water level and circulation are strongly influenced by meteorological conditions (wind and atmospheric pressure fields). Temperature in both systems can range from 0 to >25 °C, with salinity between ~18-32 PSU, decreasing along a southwest-northeast gradient.

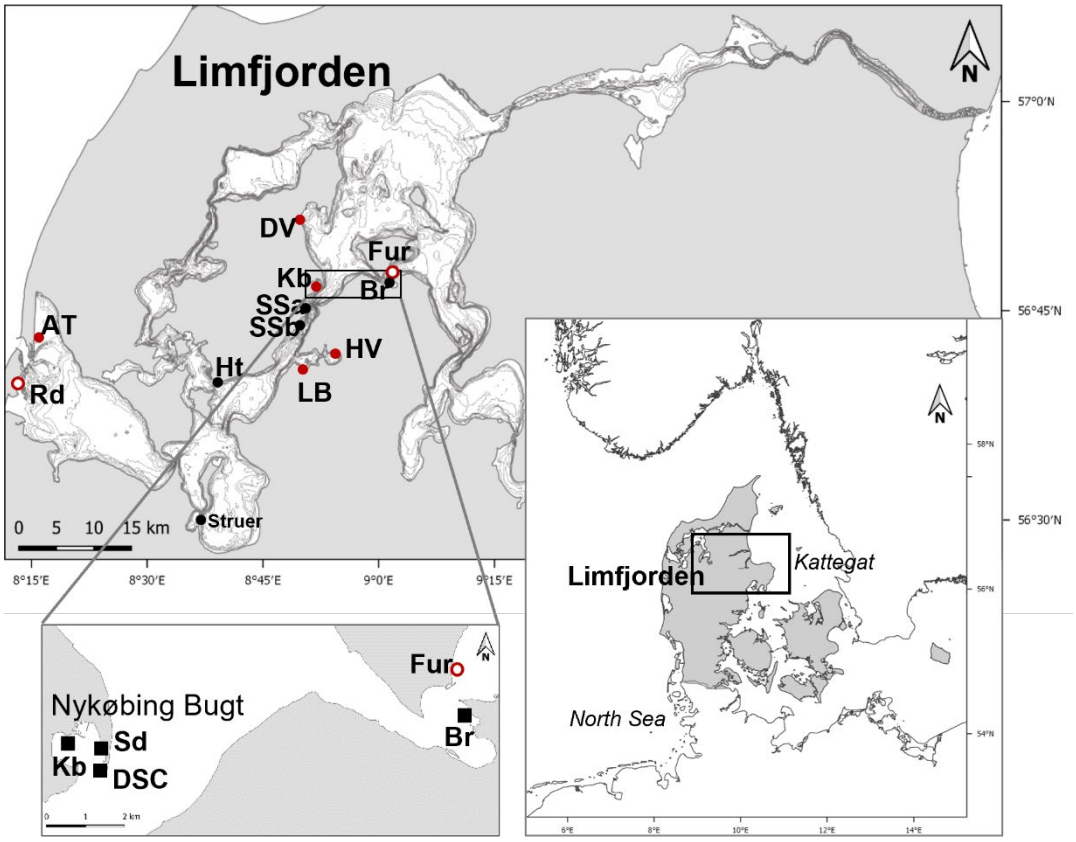

Figure 2.1.1. Location of the Limfjorden in northern Jutland, Denmark. The north-eastern side of the central basin and the eastern narrow section were not part of this study. Several sites in the Limfjorden sampled in 2006 and 2011 (Groslier et al., 2014) were also sampled in this study in 2018/2019 and 2020 (solid red circles): Agger Tange (AT), Lysen Bredning (LB), Harre Vig (HV), Klosterbugten (Kb), Dråby Vig (DV) or in 2020 only (open red circles, Rønland (Rø) and Fur). Solid black circles are additional sites sampled in 2018/2019 and 2020: Struer (St), Hesterøroddevej (Ht), Salling Sund A (SSa), Salling Sund B (SSb) and Branden (Br). Solid black squares (inset) are sites in Nykøbing Bugt and Branden sampled to evaluate mortality due to the severe 2018 winter: Klosterbugten (Kb), Strandevejen (Sd), Danish Shellfish Centre (DSC) and Branden (Br).

2.1.1 Sampling methods

Winter mortality

The 2018 severe winter impacts on Pacific oyster mortality were evaluated at three sites in Nykøbing Bugt (Klosterbugten - Kb, Strandevejen - Sd and Danish Shellfish Centre - DSC) and one site in Fur Sund (Branden - Br) known to have high densities of oysters (Figure 2.1.1). Samples were collected during winter in March 2018 and post winter in June of 2018. Three to five alongshore transects, evenly spaced between the offshore to onshore limits of the oyster populations, were randomly sampled with quadrats (50 x 50 cm, N = 3 per transect). Live oyster and dead oyster shell abundances were quantified along with any recent dead oysters, identified from the presence of decaying flesh or the pristine non-fouled inner shell surface, and interpreted as being part of the pre- winter mortality live oyster population. Mortality here is defined as the percentage change in live oyster density between winter and summer 2018.

Records of water level were obtained for Nykøbing Mors harbour, located at ca. 0.5 km from the Nykøbing Bugt and 10 km from the Branden sampling sites (www.hydrometri.dk/kommune/morsoe). Air temperature records for Morsø were obtained from the Danish Meteorological Institute (DMI: www.dmi.dk/vejrarkiv).

Deep surveys

Deep areas (>3 m water depth) were sampled in spring 2018, 2019, 2020, 2021 and 2022 (no distribution map) in up to 538 randomly distributed stations sampled using a modified oyster dredge, often in tandem or as part of annual monitoring of blue mussel and European flat oyster monitoring surveys. Track length was determined using GPS positions at the start and end of each tow. Dredge catch was sorted and weighed, separating oysters per species, blue mussels, empty shells, stones, and other invertebrates. Shell length (longest axis) was measured to the nearest 0.5 cm for all oysters in each quadrat.

The biomass of oysters (kg m^{-2}) in each fishing production area (Table 1 Appendix 2.1) was estimated using dredge track area, the total weight of oysters caught per haul (kg), total area of the fishing production areas with water depth >3 m (km^2) and applying a dredge efficiency of 33% for oysters. The sum of biomass estimates of each fishing production area estimated biomass (tons) of the entire oyster population of the Limfjorden in areas >3 m water depth.

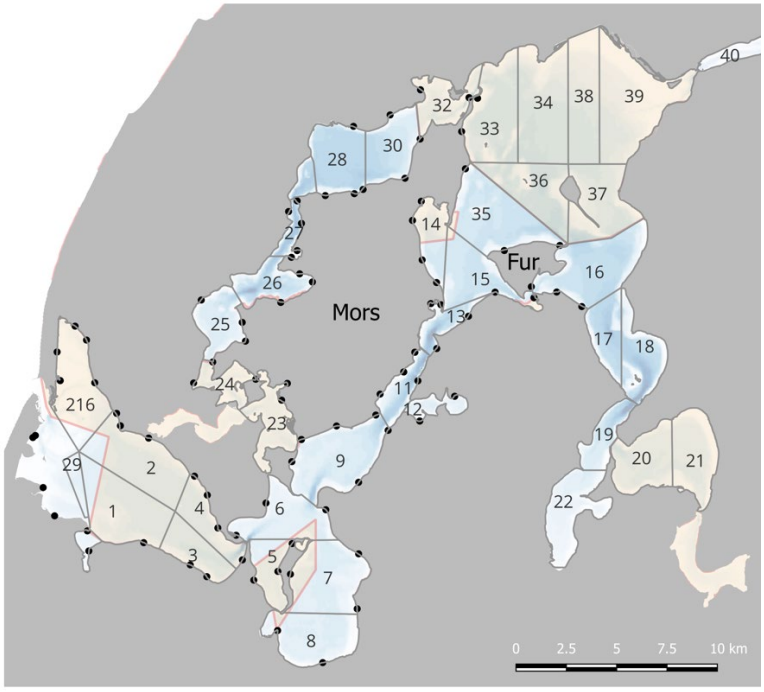

Figure 2.1.2. Bivalve fishing areas of the Limfjorden. Geographical locations mentioned in the text: Nissum Bredning (areas 1, 2, 3, 4, 29 and 216); Venø Bugt (areas 7 and 8); Salling Sund (areas 9, 11, 13 and 15); Lysen Bredning and Harre Vig (area 12); Dråby Vig (area 14); Løgstør Bredning (areas 33, 34, 35, 36, 37, 38, 39). Shaded areas are EU Natura 2000 sites.

Shore surveys

Shore surveys of Pacific oyster populations were done in late autumn 2018-early spring 2019 with 84 stations (referred as 2019 in the text) and in spring 2020 with 12 stations (Figures 2.1.1 and 2.1.2, Table 2 Appendix 2.1). The 2019 and 2020 shore surveys included sites sampled in 2006 and 2011 by Groslier et al. (2014), with four additional sites added in the 2020 survey (Figure 2.1.1).

Three to five transects were sampled at each site perpendicular to the shore and separated by ~150 m. In each transect, three to five equidistant quadrats (50 x 50 cm) were sampled depending on the transect length and maximum water level (ca. 0.5 m) that allowed sampling by hand. Live oysters within quadrats were counted, with live individuals shell length being measured to the nearest 0.5 cm and weighed. At few sites in the Limfjorden, oyster density was so low that all oysters within 5 m over the entire transect length were collected, instead of quadrat sampling. Density data is presented for oysters larger than 5 cm for comparison with data from Groslier et al. (2014).

The abundance of Pacific oysters (total number and biomass) in shallow areas in 2019 was estimated individually for the main dense beds (Branden, Klosterbugten, Harre Vig, Lysen Bredning, Struer and Agger Tange) and for the low abundance areas along Salling Sund, Fur Sund and NE coast of Mors (Figures 2.1.1 and 2.1.2). Abundance was estimated from station mean abundance, using the area occupied by individual dense beds, while for low abundance areas mean abundance per m of coast and the distance along the coast between stations was used. This estimate for shallow Pacific oyster abundance does not represent all the western and central Limfjorden, as large areas were not included. However, the estimate constitutes an initial attempt to determine the abundance of Pacific oysters in the Limfjorden, and since it includes the main populations observed in the 2019 survey it is assumed to contain most of the population.

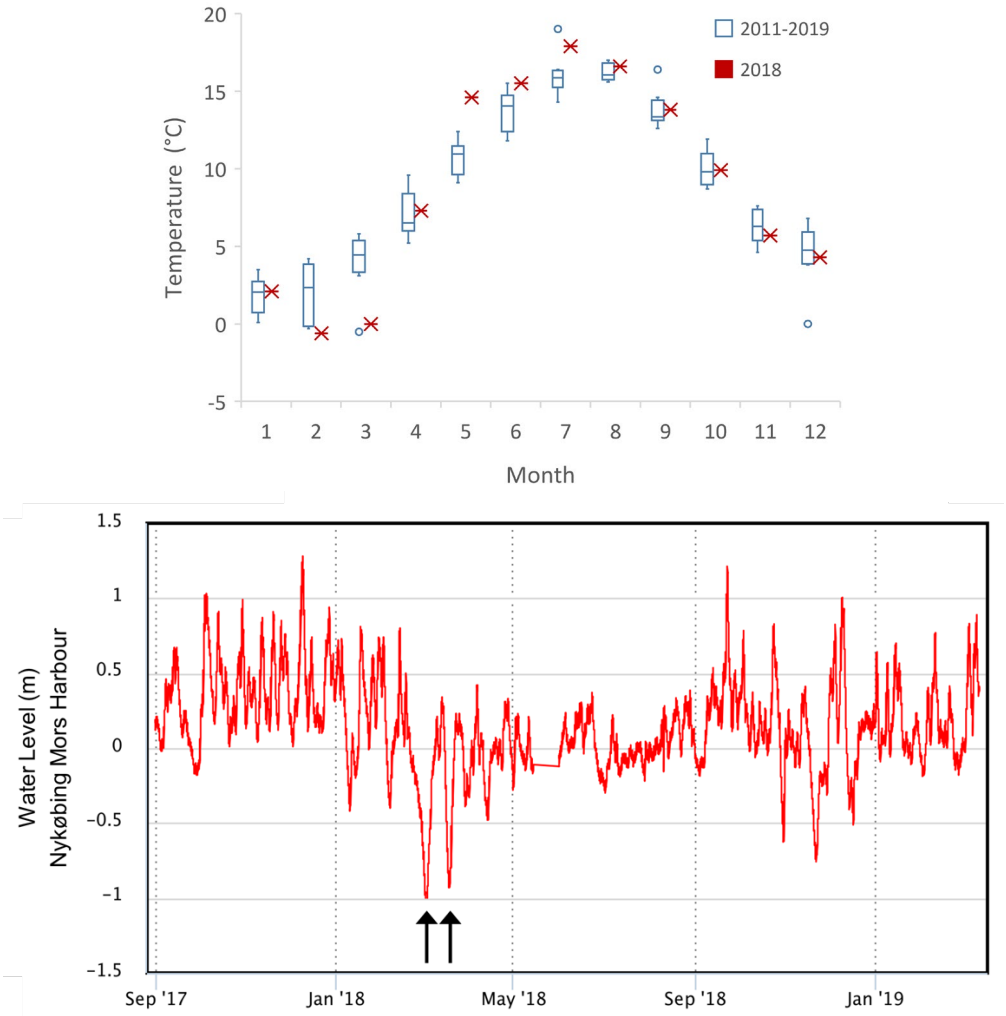

Figure 2.1.3. Top: Box plots of monthly air temperature in Morsø municipality (Danish Meteorological Institute, DMI,) from 2011 to 2019 (excluding 2018) and for 2018 (red symbols). Bottom: Water level in Nykøbing Mors harbour from September 2017 to April 2019. Arrows identify extreme low water periods in February and March 2018.

2.1.2 Results

Pacific oyster mortality due to severe winter conditions: Limfjorden in 2018

The winter of 2018 in the Limfjorden was characterized by some of the lowest February and March air temperatures since 2011 (Figure 2.1.3), with mean temperatures of -0.6 and 0 °C and minimum temperatures of -9.8 and -8.6 °C, respectively. In particular, the low air temperatures at the end of February to mid-March coincided with two periods of low water level lasting three weeks and one week (Figure 2.1.3) and sea ice formation resulting in the emersion and air exposure of vast portions of the shallow Pacific oyster populations.

In winter 2018 (March), the proportion of live oysters was higher than 90±3 % except at the DSC site with only 54±6% (Figure 2.1.4).

In the summer 2018 (June), the proportion of live oysters had significantly decreased (Z-test, $p < 0.0001$ for all) in all sites compared to the previous winter, dropping to 34±10% at Branden, 52±7% at Klosterbugten, 75±5% at Strandvejen and 22±7% at DSC (Figure 2.1.4).
Pacific oyster mortality, the percentage decrease in live oyster density between winter and summer 2018, assumed to represent the consequences of the severe winter with low temperatures and long

emersion and exposure to ice was thus 64±15% at Branden, 68±29% at Klosterbugten, 43±19% at Strandvejen and 68±19% at DSC, averaging 61±12%.

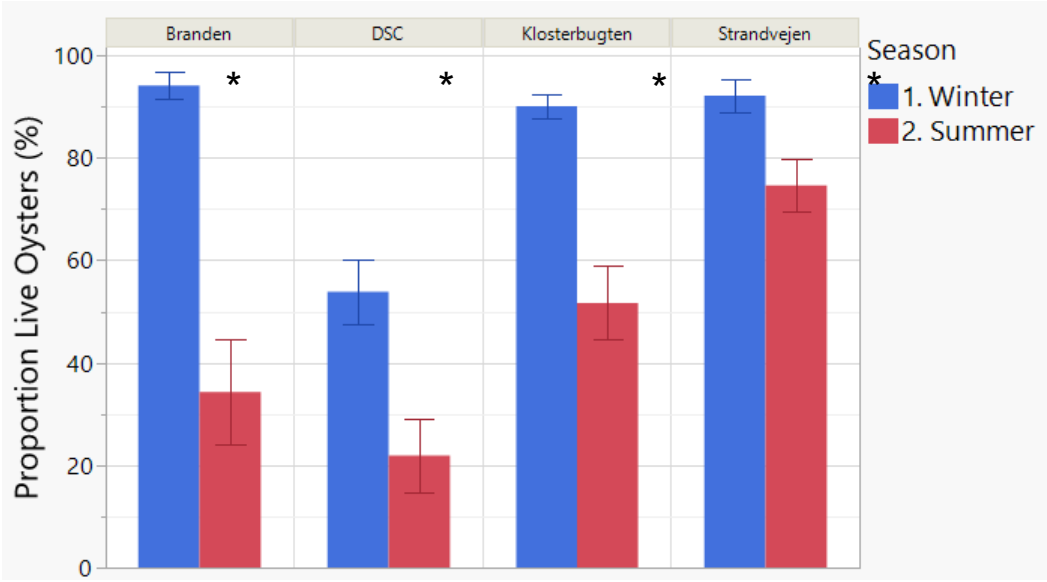

Figure 2.1.4. Proportion of live oysters Average (± SE) out of all oyster shells during winter (March) and summer (June) during 2018. Recently dead oysters where flesh remained within the shell were included as live oysters in winter. Asterisk indicates level of significance in differences: * <0.0001.

Deep areas: Abundance and distribution in 2018-2021

Pacific oysters in deep waters (i.e. >3 m) were found in most basins of the western and central Limfjorden in all years from 2018 to 2021, except in side basins such as in Thisted Bredning, Visby/Dragstrup Bredning, Skive Fjord and Lovns Bredning (Figure 2.1.5). Pacific oyster abundance was generally higher in Løgstør Bredning in the northeast, but also in Nissum Bredning in the west and Venø Bugt in the south (Figure 2.1.5). Pacific oyster biomass ranged from 0.001 to 0.606 kg/m^2 and density from 0.005 to 2.2 oysters/m^2.

No clear temporal evolution of Pacific Oyster distribution in deep waters was observed, except an increase in abundance in Venø Bugt and Kås Bredning in 2021 (Figure 2.1.5). However, total Pacific oyster population biomass in deep waters in the major basins and the Limfjorden showed a general decreasing trend from 2017 to 2022 (Figure 2.1.6). However, in Nissum Bredning Pacific oyster biomass generally remained similar from 2017 to 2020, while in Venø Bugt it increased by a factor of 1 to 10 in 2021 and 2022 (Figure 2.1.6). In contrast, in Løgstør Bredning where most Pacific oyster biomass in the Limfjorden was found, a decrease was observed from 2017 to 2022, except in 2019, also reflected for the entire Limfjorden (Figure 2.1.6).

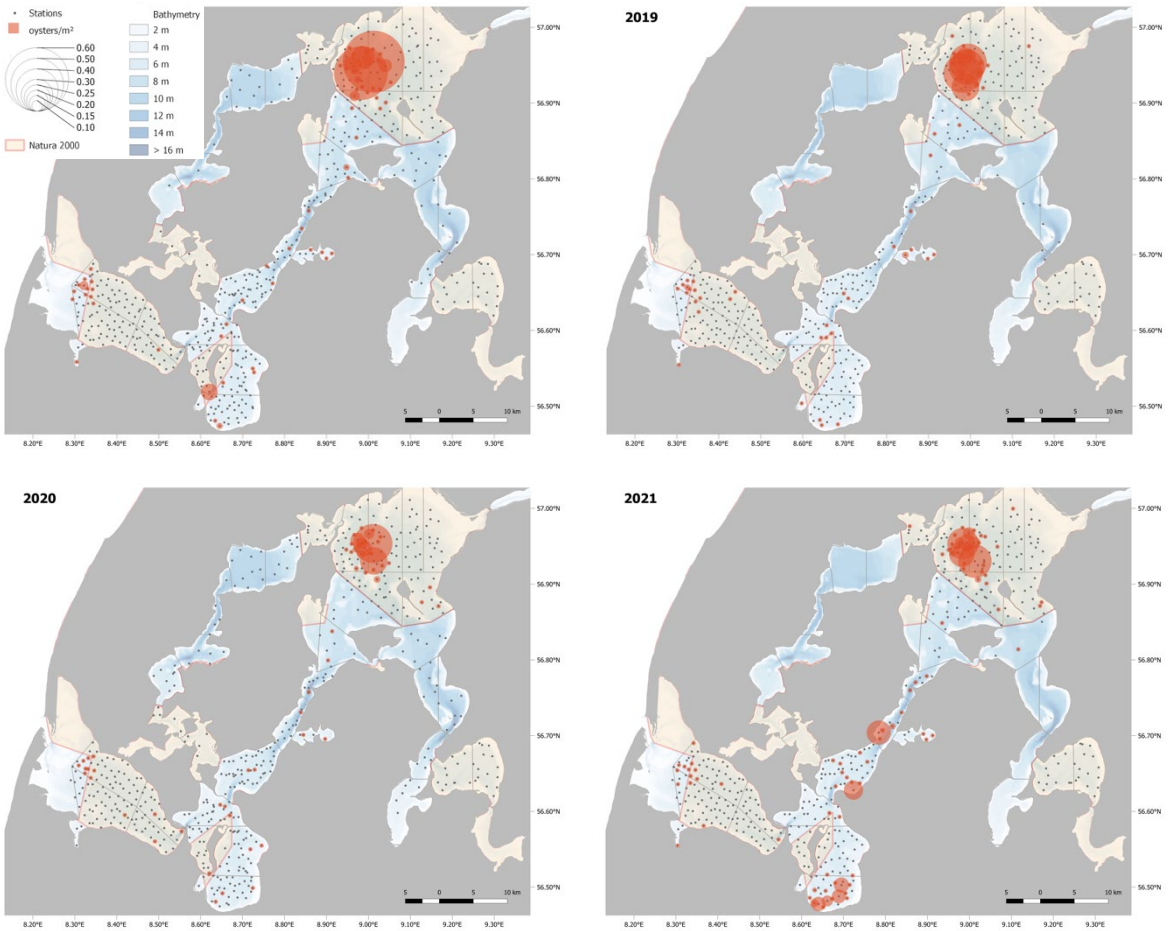

Figure 2.1.5. Live Pacific oyster density (oysters/m^2) in the deep areas of the Limfjorden in spring 2018 (top left), 2019, 2020 and 2021.

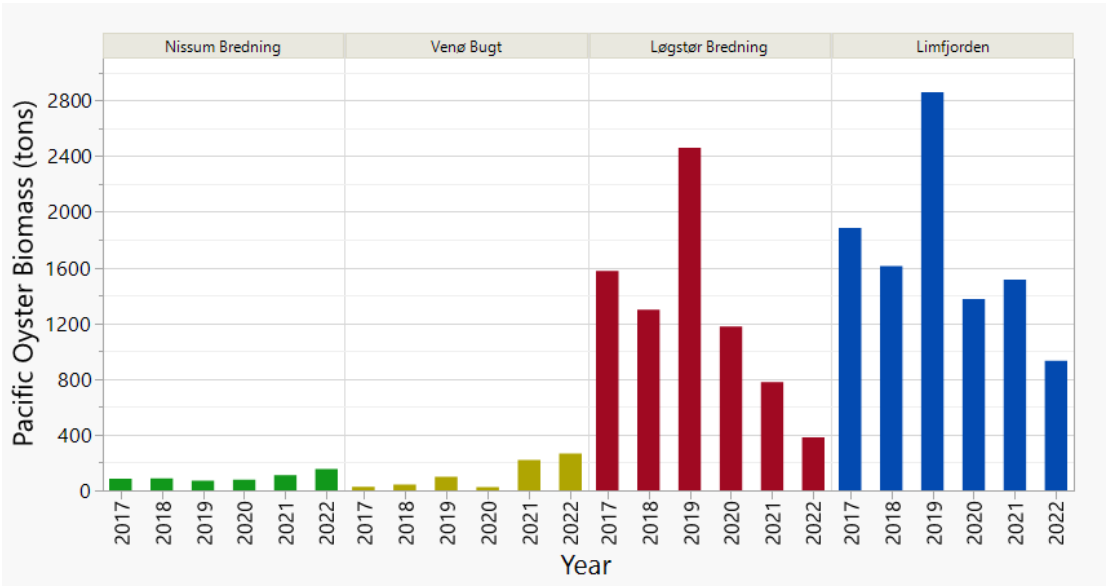

Figure 2.1.6. Pacific oyster biomass (tons) in deep areas (> 3 m depth) of Nissum Bredning, Venø Bugt, Løgstør Bredning and the entire western part of the Limfjorden from 2017 to 2022.

Shallow areas: Abundance and distribution in 2019 and 2020

In 2019, live Pacific oysters occurred at 35 of the 84 sampled sites, with most high-density sites in the central basins of the Limfjorden in Salling Sund and around the Island of Fur (Figure 2.1.7). Substantial dense populations were also present in the shores of the western and southern Limfjorden, in Nissum Bredning and Venø Bugt (Figure 2.1.7).

The highest densities occurred at Klosterbugten (79.2 ± 64.9 oysters/m^2), Fur Sund (34.8 ± 50.1 oysters/m^2), Harre Vig (29.5 ± 42.6 oysters/m^2), Agger Tange (21.9 ± 23.5 oysters/m^2), DSC (18.8 ± oysters/m^2), Lysen Bredning (14.2 ± 32.8 oysters/m^2) and Struer Havn (11.3 ± 20.9 oysters/m^2). At these sites, maximum Pacific oyster densities reached from 68 up to 270 oysters/m^2 (Figure 2.1.7).

In 2020, only 12 sites were surveyed, mainly focusing on previously sampled large dense populations to determine temporal evolution (Figure 2.1.7). Mean Pacific oyster density ranged between 2.4 and 136.3 oysters/m^2, with maximum density ranging between 12.6 and 577.6 oysters/m^2.

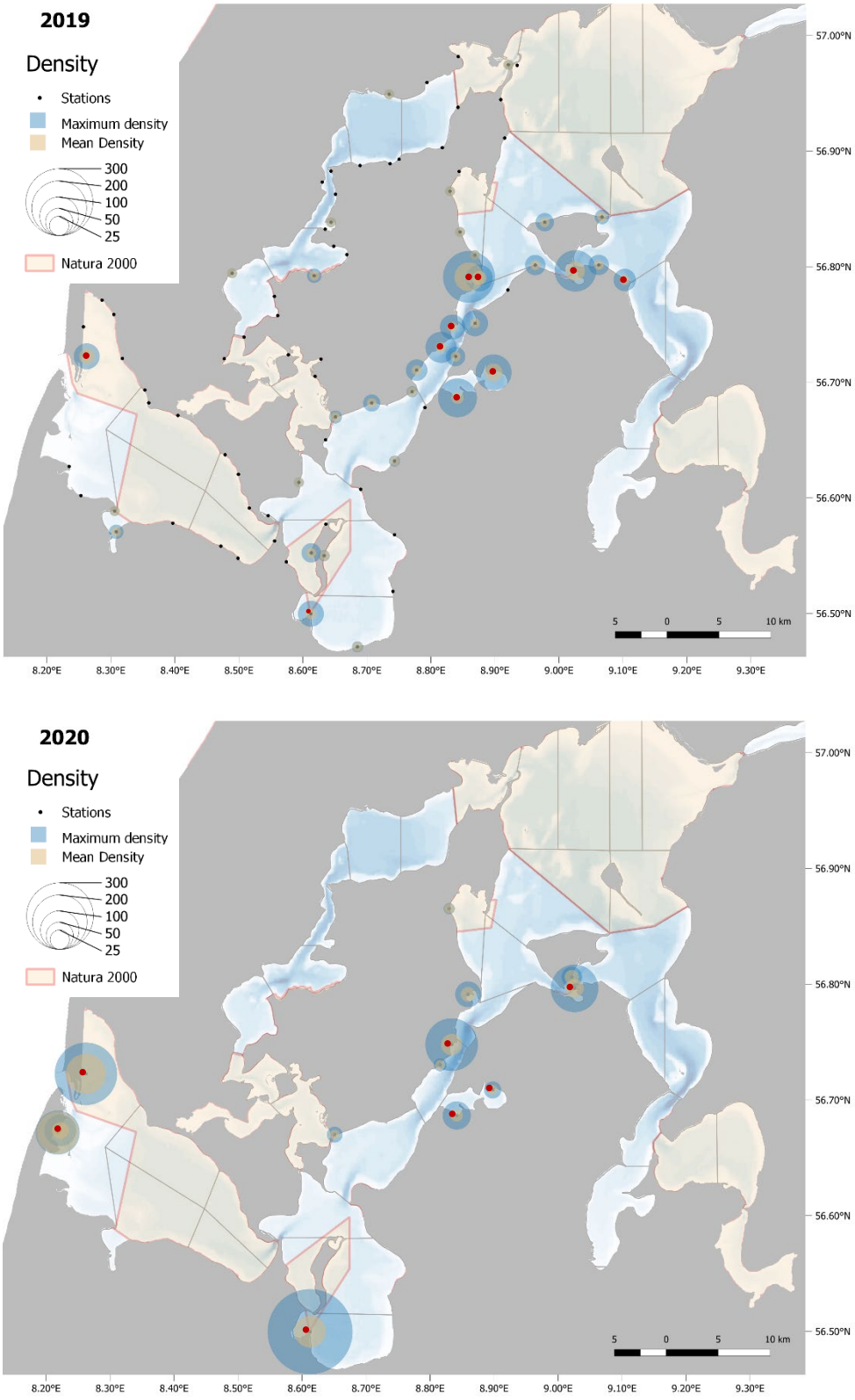

Figure 2.1.7. Mean and maximum live Pacific oyster density (oysters/m^2) in the shallow areas of the Limfjorden in autumn-winter 2018-2019 (top) and spring 2020 (bottom). Note that pie-chart scale is non-linear (function of area) for better visualization. Red circles identify sites with reef structures.

At several sites, Pacific oyster formed high-density reef structures often occupying large areas (> 1.000 m^2 at Agger Tange, Klosterbugten or Lysen) forming both littoral and sublittoral biogenic reef habitats (Figure 2.1.8).

Figure 2.1.8. Pacific oyster biogenic reefs in littoral (Agger Tange, top left) and sublittoral habitats (clockwise from top right: Klosterbugten, Branden and Lysen Bredning).

Population estimates of Pacific oysters in shallow areas are presented in Table 2.1.1. Pacific oyster population in shallow areas of the Limfjorden was estimated at 21.6 ±20.3 million oysters and 7.131 ±2.161 tons (Table 2.1.1). Dense Pacific oyster beds contained 41% of all oysters and 32% of biomass, or 14.7 ±5.4 million oysters and 2.253 ±737 tons, while low density areas contained 59% of all oysters and 68% of biomass, or 21.6 ±8.6 million oysters and 4.878 ±2.534 tons (Table 2.1.1). Single dense beds were found to contain a significant proportion of Pacific oyster abundance, with reaching over 4.5 million oysters with a biomass of higher than 500 or 1.000 tons (Lysen Bredning and Agger Tange, Table 2.1.1).

Table 2.1.1. Population estimates (± 95 CI) for Pacific oyster in 2019 in dense beds and lower density areas in shallow sites from 2006 to 2020.

		Abundance		Biomass	
	Bed Area / Coast Length	million oysters	%	tons	%
Dense Beds					
Branden	33,000 m^2	1.2±0.9	3.2	167±127	2.3
Klosterbugten	50,000 m^2	3.4±0.9	9.4	352±109	4.9
Harre Vig	24,000 m^2	0.7±0.6	1.9	69±113	1.0
Lysen Bredning	89,000 m^2	4.5±4.7	12.4	592±433	8.3
Struer	9,000 m^2	0.1±0.1	0.3	18±19	0.3
Agger Tange	221,000 m^2	4.8±2.4	13.3	1,055±561	14.8
Total		14.7±5.4	41	2,253±737	32
Low Density					
NE Mors	34,472 m	0.2±0.1	0.4	16±15	0.2
SE Mors	25,494 m	12.3±7.8	33.9	3,026±2,407	42.4
Lysen - Harre Vig	18,659 m	2.0±1.3	5.5	533±426	7.5
W Salling	31,414 m	3.7±2.6	10.2	782±549	11.0
N Salling – Fur	20,731 m	3.4±2.1	9.4	521±376	7.3
Total		21.6±8.6	59	4,878±2,534	68
All		36.4±20.3		7,131±2,161	

Timeseries of Pacific oyster abundance in the Limfjorden were obtained for several sites, some previously sampled by Groslier et al. (2014) in 2006 and 2011 (Figure 2.1.9 and Table 2.1.2), and other sites only in this study in 2018, 2019 and 2020 (Table 2.1.2). Although the large variability observed in Pacific oyster density at each site rendered differences between 2006 and 2011 relative to 2018, 2019 and 2020 years statistically non-significant (Z-test, $p > 0.05$), however density showed a clear general increasing trend, in some sites being over 100 times higher in 2019 and 2020 relative to 2006 and 2011 (Table 2.1.2).

Following the significant high mortality during winter 2018, Pacific oyster populations showed a different adjustment and evolution in 2019 to 2020, (non-parametric Kruskal Wallis test, $p < 0.05$): a decrease in Klosterbugten, remaining stable at lower density in Branden or increased to higher densities in Agger Tange (Figure 2.1.9 and Table 2.1.2).

Table 2.1.2. Timeseries of Pacific oyster density (oyster/m^2 ±standard deviation) in shallow sites from 2006 to 2020. *Density in 2006 and 2011 from Groslier et al. (2014). Evolution trend is shows by arrows. Differences in mean density at each site between 2006 and 2011 relative to 2018, 2019 and 2020 were not significant (Z-test, p > 0.05). Site acronyms as in Figure 2.1.1.

	Pacific Oyster Density (oysters/m^2)					
Site	**2006***	**2011***	**2018**	**2019**	**2020**	**Trend**
Dråby Vig (DV)	0.04±0.01	0.06±0.01		0	2.5±4.7	↑
SE Fur (Fur)		0			20.5±19.5	↑
Branden (Br)			56.3±48.5	26.4±38.0	32.9±55.6	↓=
Klosterbugten (Kb)	0.02±0.04	0.12±0.12	167.2±101.5	78.3±64.3	21.6±20.21	↑↓
Sallingsund A (SSa)				8.15±16.5	30.9±60.2	↑
Sallingsund B (SSb)				10.7±25.4	3.9±5.4	↓
Harre Vig (HV)	0.18±0.04	0.02±0.02		29.5±42.6	10.1±8.4	↑↓
Lysen Bredning (LB)	0.02±0.02	0.004±0.01		14.2±31.9	14.4±24.7	↑
Hesterøroddevej (Ht)				3.1±5.8	4.8±7.1	=
Struer (Struer)				10.8±19.8	100.1±158.3	↑
Agger Tange (AT)	3.12±1.37	0.20±0.13	99.1±108.1	21.6±23.5	76.5±48.6	↑↓↑
Rønland (Rd)		0.04±0.06			22.7±19.8	↑

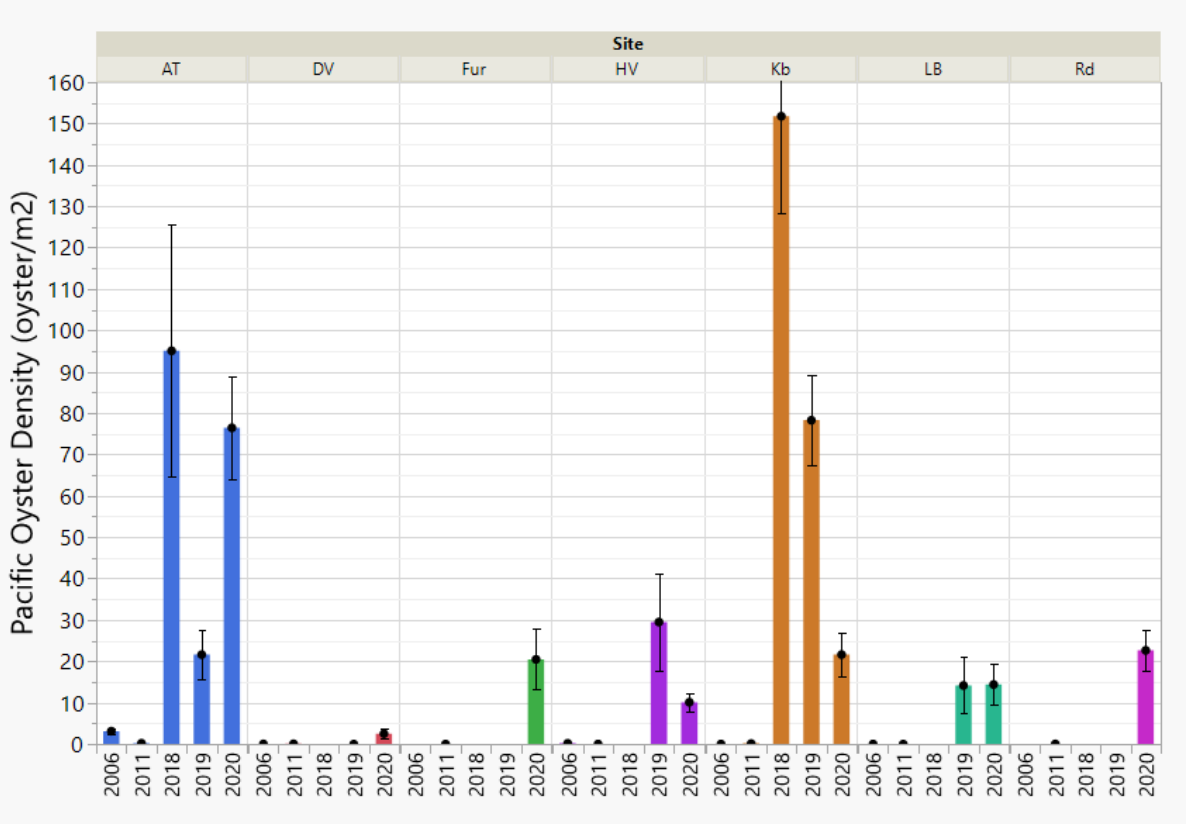

Figure 2.1.9. Timeseries of Pacific oyster density (oyster/m^2 ±SE) from 2006 to 2020 in the same shallow sites sampled by Groslier et al. (2014). Differences in mean density at each site between years were not significant (Z-test, p > 0.05). Site acronyms as in Figure 2.1.1: Agger Tange (AT), Dråby Vig (DV), Fur (Fur), Harre Vig (HV), Klosterbugten (Kb), Lysen Bredning (LB) and Rønland (Rø).

Shallow areas: Size distribution in 2019

Pacific oysters in the shallow areas of the Limfjorden in 2019 ranged in size between 0.5 and 25 cm shell length (Figure 2.1.10). The dominant size modes were centred at 3.5 to 4.5 cm and 12.5 cm in Branden, 7.5 to 9 cm in Klosterbugten, 14.5 cm in Salling Sund A, 8-11.5 cm in Harre Vig, 8 cm in Lysen Bredning, 1.5 and 14 cm in Struer and 9.5 cm in Agger Tange (Figure 2.1.10).

Size-at-age has been described for wild Pacific oysters in the Wadden Sea, the Limfjorden and the Japan Sea, its native habitat of the Pacific oyster (Cardoso et al., 2007, Diederich et al., 2006, Holm et al 2015, 2016, Kobayashi et al., 1997). Size ranges for Pacific oysters of one to five years of age, suggest two- and three-year-old cohorts dominated the Pacific oyster populations at most Limfjorden sites in 2019, reflecting successful settlement in 2016 and 2017 (Figure 2.1.10). In addition, Pacific oysters reached sizes large enough to be significantly older than five years, providing evidence for regular if not continuous recruitment (Figure 2.1.10). Significantly, recent recruitment was observed in 2019 from oysters settled in the summer of 2018, with a significant number of oysters smaller than 3 cm at several sites (Figure 2.1.10).

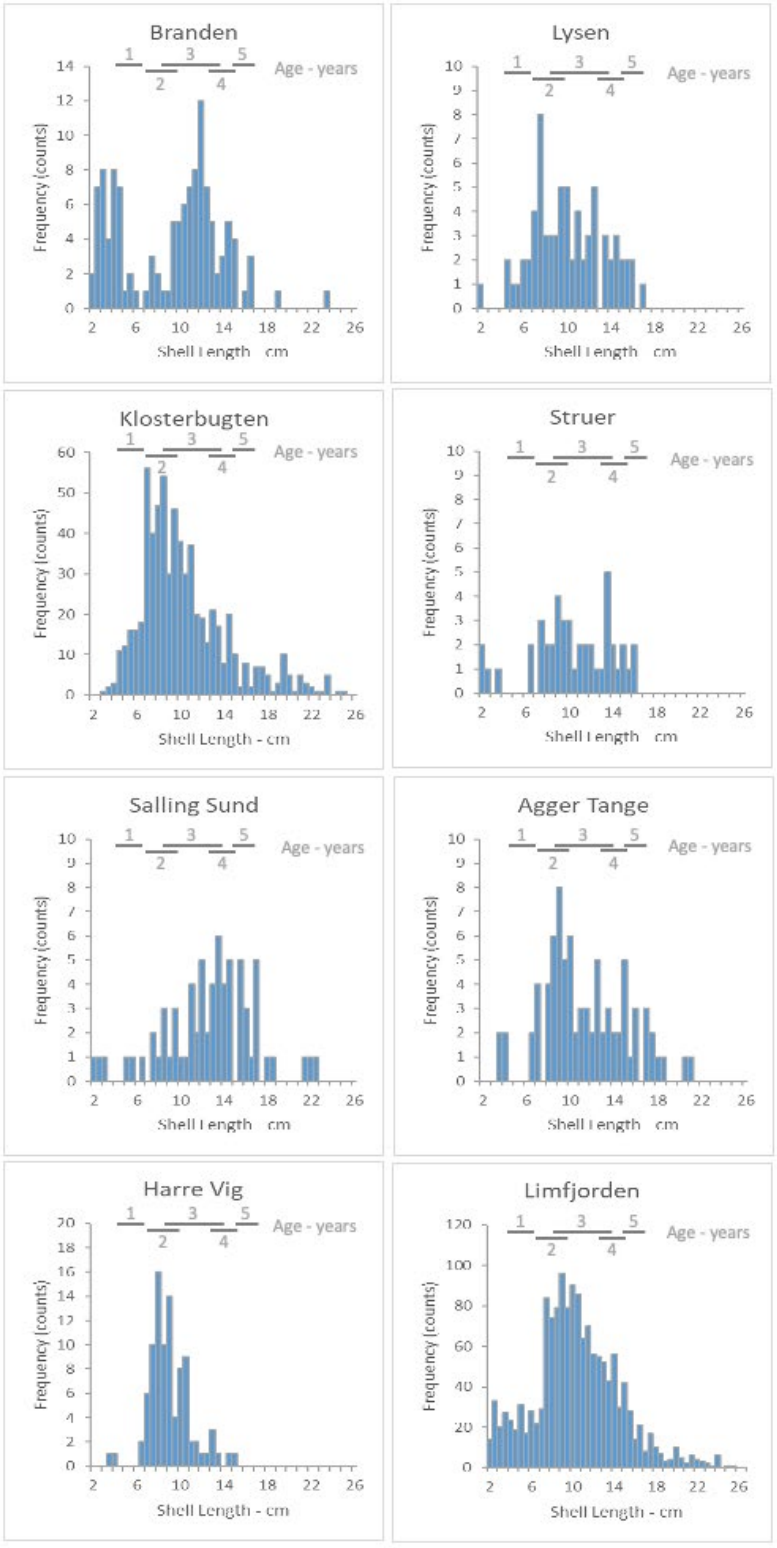

Figure 2.1.10. Pacific oyster size distribution (shell length, cm) in shallow areas of the Limfjorden in 2019: Branden, Klosterbugten, Salling Sund A, Harre Vig, Lysen Breding, Struer, Agger Tange and for the entire western part of the Limfjorden. Shown is the size-at-age range of Pacific oysters with one to five years old in the Wadden Sea, the Limfjorden and its native habitat in Japan Sea (Cardoso et al., 2007, Diederich et al., 2006, Holm et al 2015, 2016, Kobayashi et al., 1997).

2.1.3 Discussion

After over 40 years since its introduction to the Limfjorden (Kristensen, 1989; Jensen & Knudsen, 2005) and localized observations of self-sustaining and expanding wild populations (Wrange et al., 2010; Groslier et al., 2014; Holm et al., 2015, 2016), Pacific oysters are now widespread throughout the western Limfjorden both in inner basins (Løgstør Bredning, Lysen Bredning, Kås Bredning) and close to the connection to the North Sea in western part of Nissum Bredning (Figures 2.1.5 and 2.1.7). Similarly, to other areas in the North Sea and Scandinavia (e.g. Troost, 2010; Wrange et al., 2010; Laugen et al., 2015; Mortensen et al., 2017), Pacific oysters in the Limfjorden form significant feral populations in both deeper and shallow coastal habitats, albeit variable in abundance with areas having little presence, while in other areas high abundance populations often form dense reef structures.

Pacific oyster populations in the Limfjorden now reach mean densities of up to 2.2 oysters/m^2 in deeper areas (>3 m depth) and over 100 oyster/m^2 in shallow areas, with maximum densities of over 500 oyster/m^2 in oyster reefs. Pacific oysters in the deep areas of the Limfjorden ranged from approximately 1,000 and 2,800 tons with a general decreasing trend between 2017 and 2022, although increasing in Nissum Bredning and Venø Bugt, but decreasing in Løgstør Bredning where most biomass occurred.

Out of the total estimated Pacific oyster population of 10,000 tons, the major proportion occurred in shallow coastal areas of less than 1m depth, estimated at ca. 7,000 tons of which 31.5% are in dense reef containing beds and remainder in low density areas. Therefore, dense reef beds contain a significant proportion of the Pacific oyster population and are a common feature in several sections of the coast in the western and central Limfjorden likely with significant impacts on the ecosystem. The Pacific oyster is a significant ecosystem engineer, particularly when forming biogenic reef structures, causing habitat modification, increasing habitat complexity and heterogeneity, changing bottom morphology and impacting biogeochemical cycles, community composition and biodiversity (e.g. Troost 2010 and Herbert et al., 2016 for reviews).

In deeper areas of the Limfjorden, Pacific oysters were most abundant in the main fishing grounds of the European flat oyster, *Ostrea edulis*, in Nissum Bredning, Venø Bugt and Løgstør Bredning (e.g., Nielsen et al. 2019), while in shallow areas of the Limfjorden Pacific oysters co-occurs with blue mussel, *Mytilus edulis*, and with European flat oysters in eelgrass beds (this study). Therefore, it is possible that inter-species competition for habitat and food resources will occur between Pacific oysters and European flat oysters, but also with blue mussels, with potential further impacts from Pacific oyster associated diseases and co-traveller invasive species (e.g., Grizel and Héral, 1991).

In 2019 and 2020, in the shallow areas of the Limfjorden, the Pacific oyster populations were generally dominated by two- and three-year-old oysters but could reach significantly more than five years of age as indicated from size distribution. Marked differences observed in shell length distribution between the most abundant Pacific oyster populations, likely reflect variable growth rates, recruitment and mortality differences (e.g. Diederich et al., 2005; Cardoso et al., 2007; Troost 2010; Strand et al., 2012; Holm et al., 2015).

Pacific oysters in the Limfjorden can thus reach a long longevity and have frequent successful settlement even if probably not every year. Even after the high mortality of ca. 60% observed during the severe winter of 2018, successful settlement during the summer 2018 was observed. In other locations in the North Sea and Scandinavia close to its northern latitudinal distributional limit, Pacific oyster populations also show a balance between settlement and mortality, the former strongly influenced by

summer temperatures and the latter by winter temperatures and diseases (Diederich et al 2005; Troost, 2010; Wrange et al., 2010; Büttger et al., 2011; Strand et al., 2012).

A clear increase in Pacific oyster abundance in the Limfjorden was observed since 2006, with density increasing by one to four orders of magnitude from <0.01-3 oysters/m^2 in 2006 and 2011 to several 10's or even 100's oysters/m^2 in 2019 and 2020. The Pacific oyster is well established in the Limfjorden in both deep and shallow areas, but currently we do not possess enough knowledge to predict its future expansion and spread potential. As elsewhere, it can be hypothesized mild winters and warm summers will favour its expansion and spread (e.g., Troost, 2010), while disease and predation (e.g. by the Japanese oyster drill) or fishing may reduce or limit it.

To evaluate the status of the Pacific oyster population in the Limfjorden since the time of its introduction, timeseries of Pacific oyster abundance were compiled for the Limfjorden, the German and Danish Wadden Sea (Diederich et al., 2005; Büttger et al., 2011; Groslier et al., 2014; Holm et al., 2016; Vismman et al., 2016; unpublished this project). Even though Pacific oysters in the Limfjorden occurred at lower abundance by approximately 1-2 orders of magnitude, population evolution since introduction was similar to the Wadden Sea, albeit somewhat delayed by 5-10 years (Figure 2.1.11). As in the Wadden Sea (Smaal et al., 2009; Büttger et al., 2011; Reise et al., 2017), Pacific oyster population in the Limfjorden showed very low abundances after introduction, followed by a significant increase in abundance after 20-25 years, i.e. 5-10 years later than in the Wadden Sea, and finally significant fluctuations with rapid decreases and increases in abundance. Therefore, the Pacific oyster population in the Limfjorden followed the phases of invasion proposed by Reise et al. (2006) of arrival, establishment, expansion and can be considered to be in the final phase of adjustment undergoing significant fluctuations (Figure 2.1.11). Differences between the Wadden Sea and the Limfjorden in abundance and timing of population evolution are likely due to differences in habitat, environmental and biological factors affecting Pacific oyster growth rate, recruitment, mortality and thus density and biomass.

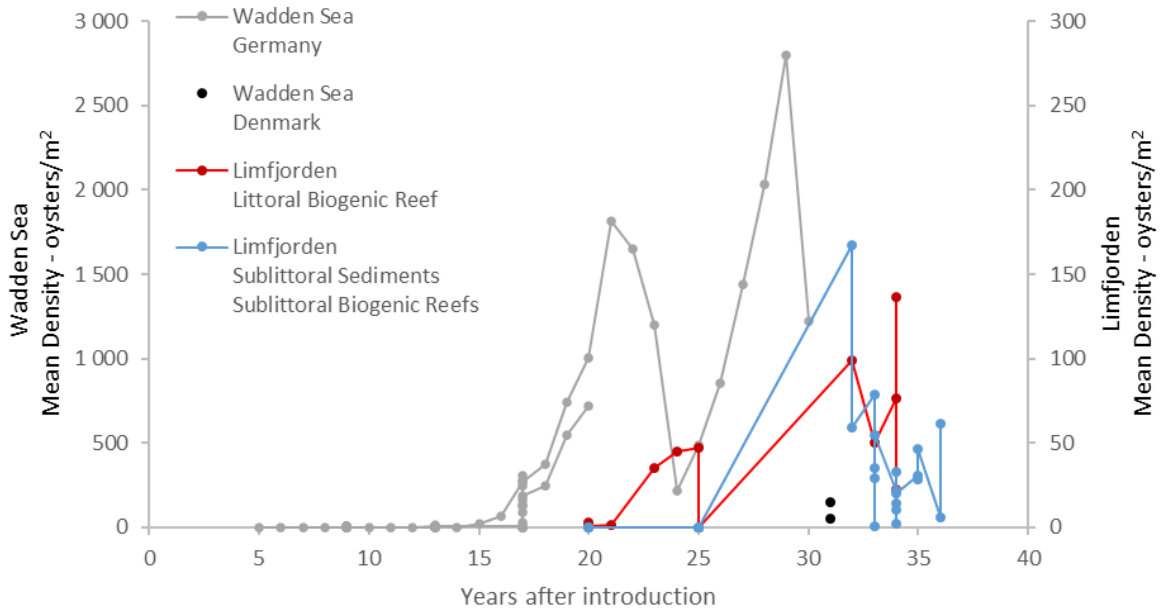

Figure 2.1.11. Evolution of Pacific oyster populations since its introduction into the Limfjorden compared with the German and Danish Wadden Sea. Limfjorden Pacific oyster populations are divided according to habitat into littoral biogenic reefs and sub-littoral or sediments. German Wadden Sea data from Diederich et al. (2005), Büttger et al. (2011), Reise et al. (2017); Danish Wadden Sea unpublished from this project; Limfjorden data from this study, Groslier et al. (2014), Holm et al. (2016) and Vismman et al. (2016).

Mortensen et al (2017) in their review considered that in Scandinavia, the habitat with the highest expansion and habitat impact risks from Pacific oysters was littoral biogenic reefs, with only low to moderate risks in sublittoral sediments and biogenic reef habitats. In the Limfjorden, only in the intertidal areas of Nissum Bredning was littoral biogenic reefs the habitat with significant Pacific oyster population expansion, often with co-existing Pacific oyster and blue mussel reefs (Figure 2.1.8, Holm 2015). However, most of the highest abundance and largest expansion of Pacific oysters in the last two decades occurred in sublittoral sediments and biogenic reef habitats in the inner microtidal areas of the fjord. These findings need to be considered when addressing the future path and evolution of the Pacific oyster in the Limfjorden, but also in other Danish and Scandinavian inner coastal waters where these habitats occur. In addition, the impacts of the large populations of Pacific oysters observed in the Limfjorden on the ecosystem, changes in habitat, community composition and biodiversity are unknown and warrant further study both at lower densities but particularly when forming biogenic reefs.

2.2 Distribution of Pacific oysters in the Isefjord

The Isefjord is ca. 36 km long and 307 km^2 in area and consists of a main large central basin connected at its northern edge through a single channel to the Kattegat and to several smaller side-basins. The average depth is ~6 m with tidal amplitude <30 cm. The Isefjord is a microtidal area, where the water level and circulation are strongly influenced by meteorological conditions (wind and atmospheric pressure fields). Temperature can range from 0 to >25 °C and with a salinity of ~16-26 PSU. Pacific oyster has been present in the Isefjord since 1986 where it was introduced through aquaculture.

2.2.1 Sampling method

In the Isefjord two surveys were undertaken in 2019 covering both deeper and shore areas. The deep areas (>3 m water depth) were sampled in spring 2019 and 120 randomly distributed stations were sampled. Dredging of four minute was carried out at each station using a modified “light mussel dredge” (width: 1m, height: 25 cm, total maximum weight: 56 kg, wire length of 50–60 m) at a towing speed of 2.5–3.5 knots. For each dredge track, the exact length was determined using GPS positions at the start and end of the tow. On board, the catches were weighed to determine total wet weight and the catch was subsequently sorted and weighed, separating Pacific oysters, blue mussels, empty shells, stones, other invertebrates and macroalgae. Furthermore, shell length was measured to the nearest 0.5 cm for all oysters and blue mussels, respectively (or a maximum of 200 individuals) at each station.

The shore survey of the Pacific oyster population was undertaken during autumn 2019 covering 34 stations. The shore survey areas were selected after asking for input on locations with Pacific oysters in online Facebook spearfishing forums reporting location with observations of Pacific oysters and previous sampling locations provided by Professor B. Hansen, Roskilde University. At each sampling station, three transects perpendicular to the shore and separated by ~150 m was sampled with equi-distant quadrats (50 x 50 cm). The length of each transects, and thus the number of quadrats, was determined by the maximum water height (ca. 0.5 m) that allowed sampling by hand. Three to five quadrats were sampled per transect to provide a minimum sample of nine quadrats per sampling site. Live and dead oysters within quadrats were counted, with live individuals shell length being measured to the nearest 0.5 cm and weighed to quantify the fresh live biomass within the quadrats. In the few stations, where oysters were observed, the density was so low that all oysters within 5 m over the entire transect length were collected. Additionally, oyster abundance was also determined within 1.5 m either side of two transects parallel to the shore between the three transects perpendicular to the shore.

2.2.2 Results and Discussion

No alive Pacific oysters were observed (Fig. 2.2.1) in the survey covering the deep areas and only one Pacific oyster shell was observed within the 120 dredge samples collected. The shore survey showed present of Pacific oysters at four stations of the 34 shore stations (Fig. 2.2.1) with mean densities of 0,0005 to 0.003 ind. m^{-2} for each transects (Table 2.2.1).

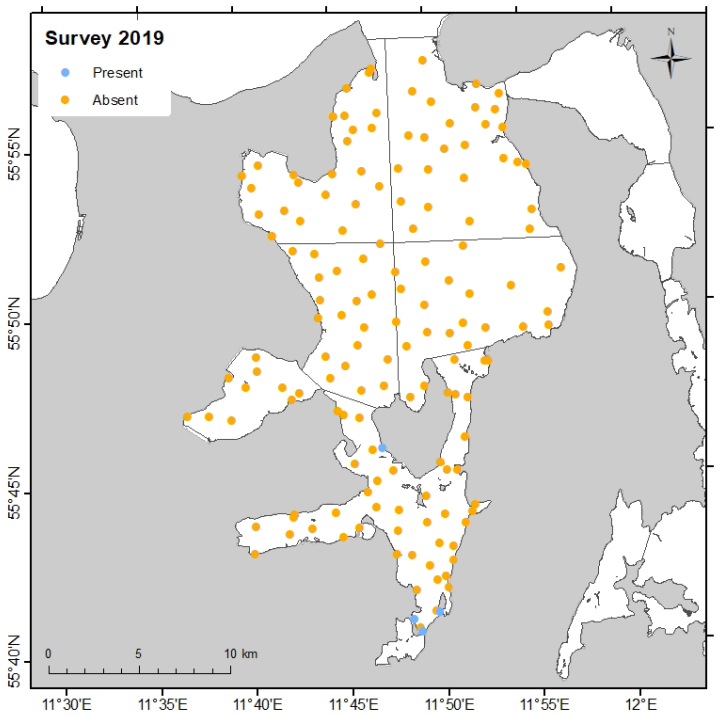

Figure 2.2.1. Observation of Pacific oysters in the Isefjord in the deep area survey (> 3m) and coastal survey carried out by DTU Aqua in 2019. A total of 154 stations were surveyed, distributed into 120 stations in deep areas and 34 stations for coastal survey.

Despite a previous study reporting Pacific oyster densities in the Isefjord in 2007 of 0.03 ind. m^{-2}, thus similar to densities in the Limfjorden at the same period in 2006 (Wang et al., 2007; Groslier et al., 2014), the Pacific oyster densities observed in 2019 were 1 to 2 orders of magnitude lower than in 2007. This indicate that the feral Pacific oyster population of the Isefjord, contrary to the Limfjorden, has not expanded after establishment. Although Pacific oyster presence in the Isefjord was continuous since introduction and thus persisting for more than two decades since initial introduction in the wild, it remains at low densities and suggests that some habitat and environmental factors are limiting its successful expansion in the fjord. As in other systems, this limitation likely arises from reduced reproduction or settlement success and/or high mortality.

Table 2.2.1. Average densities±standard deviation (individuals m^{-2}) of Pacific oysters at four shore stations in the Isefjord in 2019. The densities are calculated based on the transects sampling and not quadrates.

Transects number	Avg. density ± stdv (ind m^{-2})
1	0.003 ± 0.003
2	0.001 ± 0.001
3	0.003 ± 0.003
4	0.0005 ± 0.001
All transects	0.002 ± 0.003

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3. Testing different aerial drone mounted sensors and developing image analysis-based algorithms to optimise mapping of Pacific oysters in coastal waters

3.1 Testing different drone mounted sensors and developing image analysis-based algorithms to optimise mapping of Pacific oysters in shallow non-tidal areas

Authors: Pernille Nielsen (ed), Jonathan Gundorph, Katarzyna Gorazda, Daniel H. Olesen and Pedro S. Freitas

Mapping bivalve populations in shallow areas is often both time consuming and costly as the surveys are carried out by foot and therefore often covers smaller areas and by hand-collection of smaller quadrates. Furthermore, in the Limfjorden, the shallow (0-3 m) Pacific oyster population has an uneven distribution along the coastline both within smaller areas (a few m^2) and over longer distance (100 meters to kilometres). Aerial drones have previously been shown successful to map eelgrass beds in shallow areas of <4-5 m (Nielsen et al. 2019). However, the Pacific oysters are often white/light-coloured and might therefore be difficult to separate from other light-coloured hard substrate e.g., stones and shells or light-coloured soft sediments. Different approaches have been tested within this project and two detailed reports of the studies can be found in appendix 3.1 and 3.2. Below and overall summery of the two reports are reported.

Testing multispectral cameras and automatic detection algorithms to estimate submerged Pacific oysters

The two aims of this study were i) to test if multi-spectral cameras are suitable to map Pacific oysters in shallow waters (0-3 m) compared to an optical camera; and ii) develop good image analysis-based algorithms to segment the Pacific oysters from the background to estimate the number of oysters present in both optical and multi-spectral drone images.

The drone surveys were carried out by a DJI M-600 drone, with a mounted payload of two cameras, the MicaSense RedEdge-MX multi-spectral camera, and a standard Go-Pro RGB-camera. The two cameras were placed side-by-side (Figure 3.1.1). Consequently, two pictures, one with each camera, were taken at the same time, and from roughly the same angle, which will make a direct comparison between the two cameras feasible.

Figure 3.1.1. Setup of the two side-by-side mounted cameras. GoPro camera to the left and the MicaSense RedEdge-MX to the right (Photo: Jonathan Gundorph).

The multispectral images taken by the MicaSense RedEdge-MX camera captures data within five specific wavelength ranges (blue, green, red, red edge and near-infrared), which provide extract additional information compared to a normal RGB-camera. Each band can be separated and show different perspectives of the same area (Figure 3.1.2)

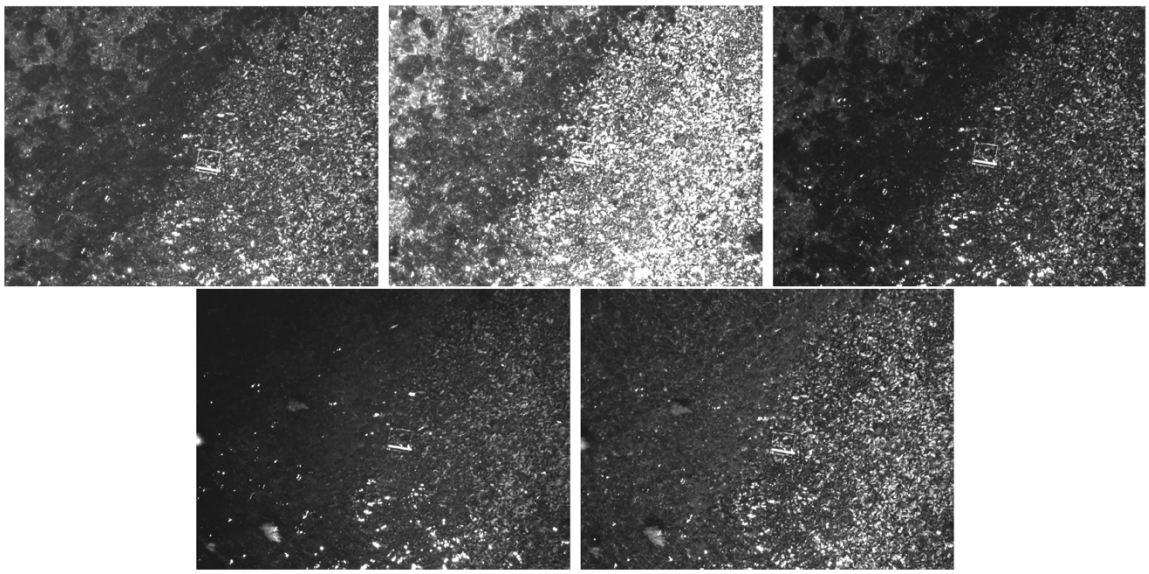

Figure 3.1.2. The images of the five spectral bands from the multispectral camera. From top left to right: Blue (475 nm), green (560 nm), red (668 nm), infrared (840 nm) and red-edge (717 nm). The Pacific oysters are shown ad white. At the centre of each of the pictures a quadrate is seen (Photo: Jonathan Gundorph).

The images from both the RGB-camera and the multispectral camera were subsequently analysed with different image analysis methods. One supervised learning algorithm named the Quadratic Maximum Likelihood classification was developed for segmentation of individual oysters, as well as two unsupervised learning algorithms named the K-means clustering, and the Gaussian Mixture Model. These three methods were tested on various pictures and evaluated for their performance to identify individual oysters. Afterwards, a semantic segmentation and an instance segmentation algorithm was developed, which managed to give a good estimate of the number of oysters present in an image under suboptimal conditions (For details see appendix 3.1). Overall, the image analysis showed that the green band of the multi-spectral camera seems to detect more oysters than in RGB-camera image, especially after computing a histogram equalization of the image.

The conclusion of this study is that a multi-spectral camera has the potential to map Pacific oysters at shallow underwater environments and may be more beneficial than RGB-camera if the right amount of post processing, the right value of gain and exposure time (ISO) of the camera, and custom configurations (like installing a circular polarized filter) are applied. Specifically for the post processing of the images to segmenting oysters, it is recommended to use the Gaussian Mixture Model, followed by an instance segmentation with a user-set value of sigma, found by trial and error, to segment and count the oysters in each image. However, the study also showed that it is not a simple protocol and would need further testing and potentially with the need for site-specific information on the environmental conditions during the drone surveys but also in relation to post images analysis.

Testing different methods to estimate the Pacific oyster coverage from drone images

The aim of this study was primarily to test different pre-processing techniques to estimate the coverage of the Pacific oysters in images captured from aerial drones. Several different algorithms were tested to segment oyster beds in the images, as segmenting oysters in images captured from above

the sea surface poses certain challenges e.g., uneven sea surface, small contrast between background and oysters, different colours of oysters and oysters covered by sand and algae.

The drone surveys were done by a DJI Phantom flying with a RGB-camera at flight hights of either 10 or 15 meters. The first step in the pre-processing of the images focused on erasing the effect of light conditions and uneven sea surface before segmentation by using different filters (low pass filter, high pass filter and notch filters) and to test different image algorithms to improve the identification of the Pacific oysters (For details see appendix 3.2).

The conclusion of this study is that the higher the flight altitude, the less details are visible in the images, which might decrease the segmentation results. However, the available datasets did not provide enough information to draw additional conclusions. Improved visibility underneath the water surface was obtained by using low pass filter as the contrast was significantly enhanced. On the other hand, using lowpass filter might blur the contours of the individual oysters and thereby decrease the segmentation success rate. Using the dehazing algorithm generally improved the visibility (Figure 3.1.3). Overall, the segmentation of oyster beds has proven to be challenging and none of the tested algorithms have provided satisfactory results, which is caused by different environmental conditions e.g., light and wave conditions, vegetation within and upon the Pacific oysters, difficulties in segmentation of Pacific oysters from background (bottom) colours, where especially individual oysters are difficult to segment.

Segmentation of oysters is so dependent on the environmental changes it is recommended to i) capture similar images in the same area during different seasons; ii) focus on neutralizing different lighting conditions e.g., Retinex algorithm and iii) investigate supervised learning to develop a successful segmentation. It will require more work to create and annotate a representative dataset, but certain characteristics of oysters (e.g., similar values in saturation) were observed in different images and might be used as an indication of presence of Pacific oysters.

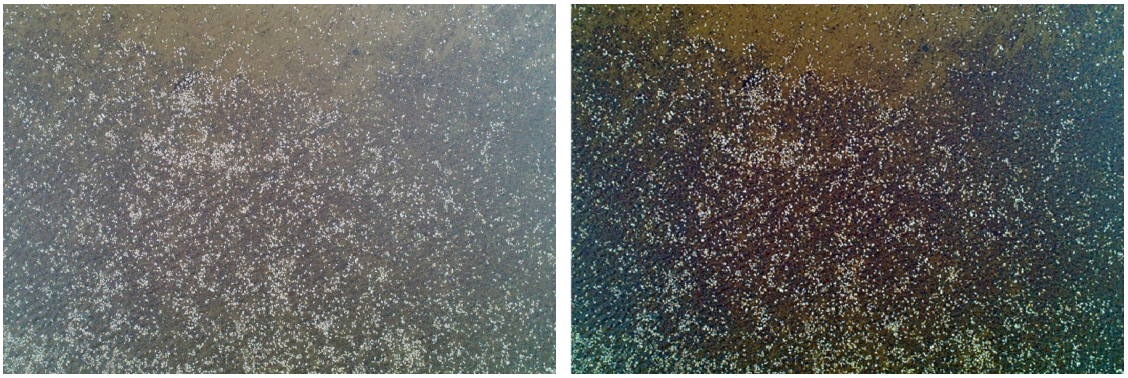

Figure 3.1.3. Original (left) and dehazed filtered (right) images.

3.2 Testing the use of drone mounted LiDAR scanner for mapping bivalve beds at low tide

Authors: Pernille Nielsen and Kerstin Geitner

Mapping of bivalve beds in tidal areas are often time consuming and costly due to the hand-collection of e.g., quadrates in the tidal areas. Furthermore, the biomass estimation is challenged by the uncertainties of extrapolating relatively small quadrates to entire bed sizes with often a very patch distribution. In the Wadden Sea, initial experiments with aerial drones mounted with a RGB camara showed that drones contribute to a more efficient and systematic mapping of bivalve beds in tidal areas, which improved the biomass estimation (Nielsen et al. 2018). However, the density of the bivalve beds varies across the beds (Figure 3.2.1), which can be difficult to identify from photos taken from drones at a flight hight of 20-90 m. Using a Light Detection and Ranging (LiDAR) scanner instead of an RGB camera the 3D structure of bed is mapped and thereby the differences in densities of the bivalves across the mussel bed can potentially be accounted for in the biomass estimations.

Figure 3.2.1. Left: High densities of blue mussels/Pacific oysters. Right: Low density of blue mussels/Pacific oysters at the same bed (Photos: Pernille Nielsen).

Selection of area and collection of ground truth data

Based on the previously drone studies (Nielsen et al. 2018), an area known to have multiple dense mixed beds of blue mussels and Pacific oysters were selected for the LiDAR-test (Figure 3.2.2). Within this area, one bed was randomly selected for ground truth sampling to be able to estimate separate total biomass of blue mussels or Pacific oysters as either kg m^{-2} or kg m^{-3}. Consequently, before the drone flight, 14 quadrates (0.25 x 0.25 m) were collected along the entire length of the bed with 4-5 m between each quadrate. The sampling location of each quadrate was determined by marking the GPS position. Within each quadrate, the top layer of approximately ten centimetres of the bed, where the alive species are observed, was collected. The samples were brought to land and weighed to determine total wet weight and the catch was subsequently sorted and weighed, separating Pacific oysters, blue mussels, empty shells, stones, other epifauna and macroalgae. Furthermore, the shell length of blue mussels and of pacific oysters was measured to the nearest 0.5 cm for all blue mussels and Pacific oysters (or a maximum of 200 individuals) for each quadrate.

Figure 3.2.2. Drone photo of multiple mixed beds of blue mussel and Pacific oysters near Sædding Strand, September 2021. The bed mapped with LiDAR is marked with pools at the ends and with white buckets randomly positioned across the entire length of the selected bed (Photo: Drone pilot Alexander Rietz Vesterhauge).

Drone payload and flight information

The LiDAR-scanner (payload package) was an EagleScanner X1 developed by Droneinnovator. The EagleScanner X1 consists of three sensors: i) a LiDAR horizon sensor from Livox providing 240.000 points per second; ii) a Global Navigation Satellite System (GNSS) receiver (Tersus BX316D real time kinematic RTK board); and iii) an Inertial Measurement Unit (IMU) (Xsens MTi 100). The LiDAR-scanner was mounted on a DJI M600 drone flown at a flight height of 30 m above the area of interest and with a speed of approx. 7 m/s. Accurate and precise navigation were based on post-processed high precision GNSS and IMU data in a tightly coupled Kalman filter solution with forward and backward calculation.

Data treatment

The geo-referenced LiDAR data was used to calculate the volume and the area of the selected mixed mussel bed. To achieve this, a 3D point cloud processing software was used, and two methods were implemented: i) identify neighbour points around the bank that represent sea level (points that did not penetrate through water) and ii) identify neighbour points around the bank that represent bottom level (points that managed to penetrate through water and represent the bottom surface under the sea). For each method, the interpolation method was used to produce a 3D model (method 1) that best represents the water and bottom level respectively and then combined with the ground truth biomass data for the blue mussels and pacific oysters at each sampling position to calculate the biomass of each species (kg) using the formula $m = \rho A \times A$ (m = mass, A = total are and ρA = average area density) and the volume density of each species (kg m^{-3}) using the formula $\rho = m/V$ (m = mass, V = total volume, ρ = volume density) for the bottom surface, as this method would give the most accurate estimate of the entire biomass of the two species of the selected bed.

The LiDAR data was also analysed by using ArcGIS. First the ET GeoWizard Extension for ArcGIS desktop, using a threshold of 0.2 meters were used to create the boundary of the area of the mixed mussel bed, followed by an inverse distance weighted (IDW) interpolation with default values and with a cell size of the output grid of 10x10 cm to visualize the differences in height of the mixed mussel bed. Correlation analysis (Spearman correlation, $p = 0.05$, Prism 9.4.0 for Mac) of hight of the mixed mussel bed and the biomass (kg m^{-2}) of each of the two bivalve species were also examined by either

i) using the nearest LiDAR point to the ground truth (quadrate) sampling station in question to assign a height, or ii) using the interpolated grid data value directly beneath the sampling station.

The total biomass (kg) of each of the two bivalve species were estimated by two different methods; i) biomass estimations were performed separately for four depth intervals (<0.3, 0.3-0.4, 0.4-0.5 and >0.5 m), where the lowest and the highest intervals were merged due to lack or very few stations in these intervals. For each depth interval, the area was calculated from the interpolated grid, derived from the LiDAR data, and multiplied with the average density of either blue mussel or Pacific oysters at the specific heights (method 2) or ii) the total area (m^2) of the bed was calculated and multiplied with the average density (kg m^{-2}) of blue mussel and Pacific oyster, respectively (method 3). The area at different depth intervals varied, consequently the biomass for each depth interval were normalised to the total area of the mixed mussel bed (896 m^2). Furthermore, the average shell length (±sd) of the Pacific oysters and of blue mussels at each depth interval were estimated and significant differences were analysed by (One-way ANOVA, followed by Tukey's multiple comparison test, p = 0.05, Prism 9.4.0 for Mac).

Results and discussion

The results of the interpolation from either sea level or from the bottom showed that the larges area of the mixed mussel bed is obtained by using the bottom surface compared to the sea surface (Figure 3.2.3). The areas using either the bottom or sea surface interpolation method were estimated to 862 and 836 m^2, respectably corresponding to a volume of 417 m^3 and 208 m^3.

In the tidal areas in the northern part of the Wadden Sea, a patchy distributed array of multiple mixed mussel beds, where each bed is clearly distinct, occur along the shoreline for several kilometres (Figure 3.2.2). The patchy distribution of distinct mixed mussel beds can result in overestimations of the total bivalve populations when interpolating from individual beds to a total population in a larger area if the patchy distribution is not considered in the interpolations (Further details see Nielsen et al. 2018). Furthermore, the hight of the mussel bed vary from a few centimetres and up to >80 centimetres (Figure 3.2.3), where the highest points are typically at the centre of the bed and decreasing towards the edge of the bed. The differences in hight of the mixed mussel bed could potentially reflect different densities of the two bivalve species across the mixed mussel bed (Figure 3.2.4).

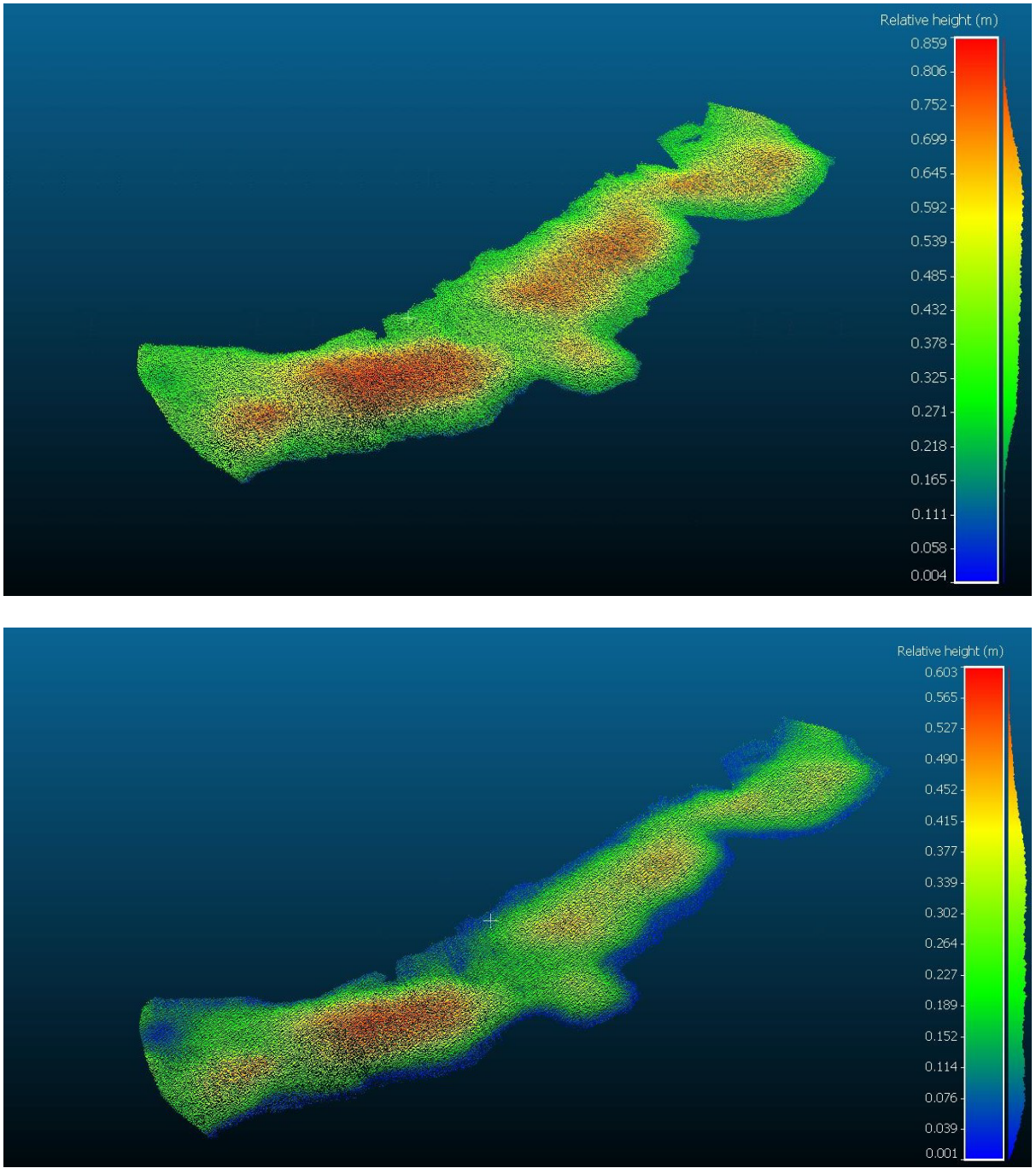

Figure 3.2.3. LiDAR interpolated data showing the relative height of the mixed mussel bed from the bottom surface (top) and sea surface (bottom).

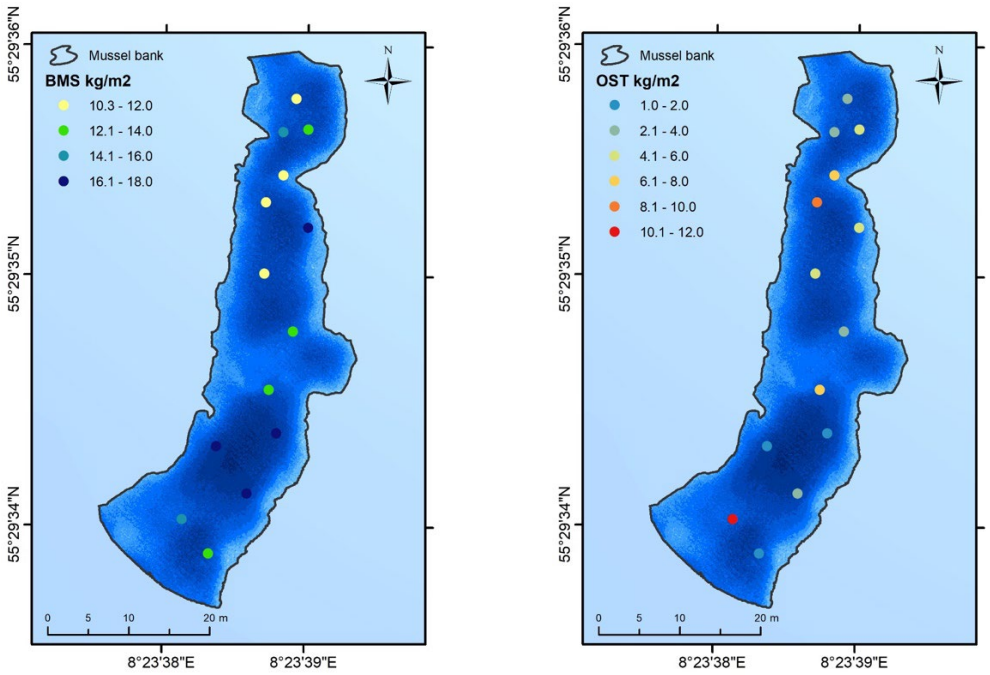

Figure 3.2.4. ArcGIS IDW interpolated LiDAR point data, where darker blue colours indicate the highest elevation above the sea bottom. The dots show density (kg m^{-2}) of blue mussels (left) and Pacific oyster (right) in the collected quadrates.

The total biomass of blue mussels and Pacific oysters estimated with three different interpolations varies from 11,800 to 12,265 kg and 4,192 to 4,733 kg, respectively (Table 3.2.1). The differences between the biomass estimated with the 3D model interpolation (862 m^2, Method 1) and the GIS-interpolations (896 m^2, method 2 and 3) were due to the slightly larger (~4%) estimated area of the mixed mussel bed in the GIS interpolation (Table 3.2.1). The variation between the two GIS-interpolation estimation of the biomass for blue mussels and Pacific oysters varied with approximately 1% and 8%, respectively. Overall, the different interpolations gave similar biomass estimations of the two bivalves species when using the same LiDAR data.

Table 3.2.1. Estimated areas (m^2) and biomass (kg) of blue mussels and Pacific oysters using different interpolations of LiDAR data. Method 1: 3D-model, Method 2: GIS depth integrated and Method 3: GIS total area integration (see text for more details).

	Method 1			Method 2			Method 3		
Height interval (m)	Area (m^2)	Mussel biomass (kg)	Oyster biomass (kg)	Area (m^2)	Mussel biomass (kg)	Oyster biomass (kg)	Area (m^2)	Mussel biomass (kg)	Oyster biomass (kg)
<0.3	-	-	-	99	1,662	223	-	-	-
0.3-0.4	-	-	-	200	2,663	1,158	-	-	-
0.4-0.5	-	-	-	207	2,429	1,072	-	-	-
>0.5	-	-	-	390	5,400	2,281	-	-	-
Total	**862**	**11,800**	**4,192**	**896**	**12,154**	**4,733**	**896**	**12,265**	**4,357**

Neither the biomass of blue mussels nor Pacific oysters estimated for each depth interval correlated with the height of the mixed mussel bed ($p > 0.05$, data not shown) and similar for the depth interval normalised biomass data ($p > 0.05$, Figure 3.2.5).

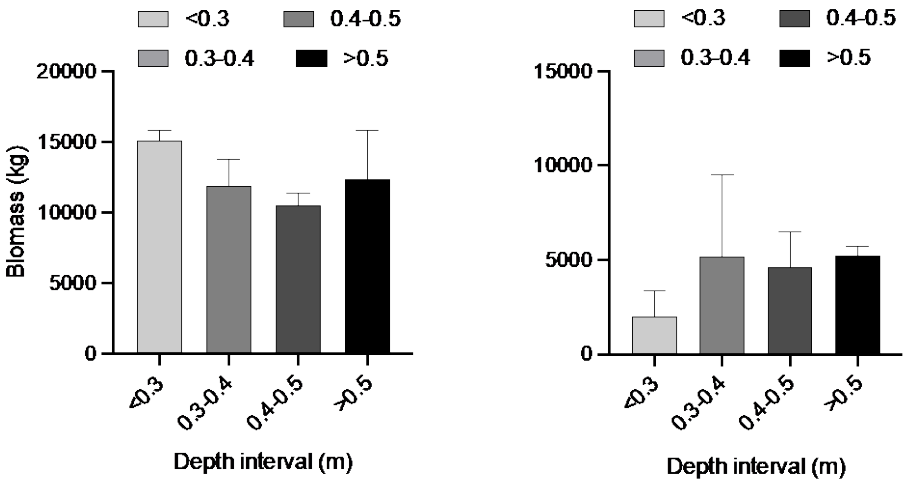

Figure 3.2.5. Normalised biomass estimations of blue mussels (left) and Pacific oyster (right) at different depth intervals. Note different y-scale.

The average shell length of blue mussels and of Pacific oysters for each depth intervals were not significantly different ($p > 0.05$, Figure 3.2.6).

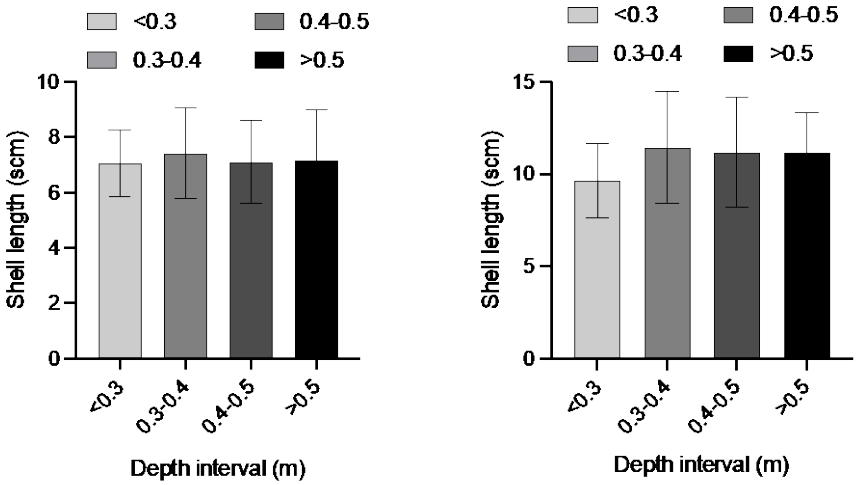

Figure 3.2.6. Average shell length ±sd of blue mussels (left) and shell length ±sd of Pacific oyster (right) in semi-centimetres (scm) at different depth intervals. Note different y-scale.

The analysis of the biomass of blue mussels and Pacific oysters and shell length respectively did not correlate or vary with height of the mixed mussel bed. However, the analysis was based on the 14 ground truth samples, which were collected along a middle transect (Figure 3.2.4) and did not include samples collected at the edges of the bed. For the Pacific oyster the mean biomass was lower at <0.3 m (Figure 3.2.5) and had smaller oysters (Figure 3.2.6) in the depth interval <0.3 m compared to the other depth intervals, but it was not statistically significant.

3.3 Conclusion for using different drone mounted sensors and developing image analysis-based algorithms

The use of aerial drones mounted with different sensors (e.g., RGB-camera, multispectral sensor, or LiDAR sensors) to map Pacific oysters in either shallow waters in the Limfjorden or in tidal areas at low tide e.g., the Wadden Sea is fast and can cover relatively larger areas in terms of mapping the extent of the reefs and individual oysters. However, it becomes more time consuming and difficult to either quantify the total biomass or coverage of the oysters as the post processing of the drone images are challenged by site specific and environmental conditions. Consequently, the post processing of the drone images to estimate the Pacific oyster biomass or coverage would require further optimisation in relation to adjustment of sensor configurations, surveying the same area during different seasons and neutralizing different lighting conditions in post processing of the images to potentially be able to develop a successful segmentation of the oysters by supervised learning. This will require a larger and more representative dataset compared to the dataset used in this study.

Mapping the 3D structure of the mixed mussel bed by using the LiDAR sensor provide detailed information about the height dynamics of the bed, which varies from a few centimetres at the edge of the bed to >0.5 m at the centre but also vary along the length of bed (Figure 3.2.3). Using different interpolation methods to estimate the biomass of blue mussels and Pacific oysters showed similar results (Table 3.2.1) and no significant correlation was observed between biomass densities (kg m^{-2}) and the height of the bed, this could be due to the relative low ground truth sampling frequency (14 frame samples) mainly located along the length axis of the mixed mussel bed (Figure 3.2.4). A high frequency ground truth sampling of the mapped mixed mussel bed is recommended to examine if the hight of the mixed mussel bed correlates with the biomass (kg m^{-2}) or size of either blue mussels or Pacific oysters.

Base on the experiences within this project using different sensors mounted on aerial drones and different post processing image analysis, the challenges with estimation of oyster biomass is mainly due to insufficient post processing image analysis (further details see above) and the need for adjustment of sensor configurations to fit the site-specific conditions. If these issues can be solved by machine learning of larger datasets, then the use of aerial drones to map Pacific oyster populations (likely also other bivalves) in shallow areas can increased both the areas mapped, likely at a lower cost and improve the biomass estimation.

3.4 References

Nielsen MM, Linden-Vørnle M & Petersen JK (2019). Forvaltningsgrundlag for fiskeri af muslinger i Natura 2000-områderne Horsens Fjord og Lillebælt. DTU Aqua-rapport nr. 343-2019. Institut for Akvatiske Ressourcer, Danmarks Tekniske Universitet. 28 pp. + bilag.

Nielsen P, Geitner K, Jakobsen J, Köppl CJ, & Petersen JK (2018). Fagligt grundlag for forvaltningsplan for udvikling af bæredygtige fiskerier af muslinger og østers i Vadehavet. DTU Aqua-rapport nr. 334-2018. Institut for Akvatiske Ressourcer, Danmarks Tekniske Universitet. 33 pp. + bilag.

4. Genetic characterization of the Pacific oyster population of Danish waters

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When non-native species are introduced in the wild, they can adapt to the local environment and compete for resources with other species, potentially posing a negative impact for the ecological balance of the ecosystem where the species establishes itself (Wrange et al., 2010). The control of invasive species is therefore needed in many instances to maintain a healthy ecosystem. One way to control an invasive species, such as the Pacific oyster in Denmark, is the establishment of a fishery or controlled catches, for which the knowledge of the presence of the species and its density is of interest. However, coupled with density information is the knowledge about the genetic status of the population under study, which is currently unknown for Pacific oyster populations in Danish waters, and is essential from the point of view of mitigation and restoration actions regarding the management of an invasive species. For instance, it is unknown how many different genetic stocks (i.e. populations whose individuals share genetic material between them) are there in the Limfjorden and the Isefjord. Pacific oysters were imported in connection with aquaculture activities to Denmark on several occasions, but whether the individuals now found in Danish waters belong to different settlement events that occurred over the period from the 1970s to 2000 where aquaculture activities in Denmark occurred (Nehls & Büttger 2007) is not known, i.e. do they come from the same or several source populations. There is also a lack of information on how recruitment to and spread from the Danish stocks takes place from a genetic point of view (gene flow). Likewise, it is not known whether spatial genetic variation of the Pacific oysters (if any) is due to the stocks being genetically adapted to different environmental conditions, i.e. local adaptation, which can provide a population with genetic characteristics that make them thrive 'better' in a certain environment.

Based on this background, the purpose of the genetic studies within the project was: (1) to characterize the stock structure of the Pacific oysters in the different water sections in the Limfjorden and the Isefjord, (2) to determine the genetic relationship among them, i.e. determining whether the stocks in the different water sections are independent populations or else they spread freely between the different areas, and (3) study the potential local adaptation of the stocks situated in Danish waters. We specifically aimed to investigate genetic variability at a small spatio-temporal scale by collecting samples at the same locations from different size/age cohorts. To achieve these objectives, we started by first developing a panel of Single-Nucleotide Polymorphism[2] (SNP) DNA markers, a type of genetic markers that can provide information about separate stocks and an indication of whether the stocks possess specific environmental adaptations, using information from a previous study on Pacific oysters (Vendrami et al., 2019). By analysing ~1200 individuals collected within the project from different areas in Danish waters, together with three foreign stocks (the Netherlands, Sweden, Norway), it has resulted in the characterization at the genetic level of the population of Pacific oysters in Danish waters, and the understanding of the establishment history and spread of stocks from a genetic point of view.

[2] A base pair (C, T, G, A) in the DNA where there is variation within individuals of a population

4.1 Methods

Collection of samples

We collected samples between 2016 and 2021 at different locations in the Limfjorden in Jutland, as well as Wadden Sea and Isefjord (Zealand). The coordinates from the different sample locations are presented in Table 1. For one of the locations ("Lysen") we sampled both in shallow water, and deeper (1-3 m), just offshore of the shallow bed ("Lysen offshore"), and we kept those two samples as independent location samples. Shells were measured for width and length. Tissue samples were taken from each individual and stored in ethanol. We also included samples from a single location from the Netherlands, Norway and Sweden; these samples were collected by local researchers, who measured the shells, extracted a tissue sample, and sent the sample stored in ethanol to DTU facilities. A total of 1182 individuals were processed in the genetic laboratory at DTU in Silkeborg. The number of samples per location ranged from a minimum of 28 (Isefjord) to 147 (Wadden Sea) (mean=73.87, median=67) (Table 4.1.1).

Table 4.1.1. Coordinates (longitude/latitude) of the localities where samples were obtained, together with the number of samples.

Country	Locality name (zone no.)	Longitude	Latitude	Sample size
Denmark	Agger Tange (216)	8.25722	56.7209	103
Denmark	Struer (8)	8.61219	56.4997	88
Denmark	Sallingsund (13)	8.87517	56.7429	73
Denmark	Nykøbing Bugt (13)	8.87517	56.7899	61
Denmark	Branden (15)	9.02607	56.7971	101
Denmark	Dråby (14)	8.83471	56.8735	68
Denmark	Nissum (NA)	8.19171	56.6271	59
Denmark	Løgstør (33)	8.59297	56.5529	40
Denmark	Isefjord (110)	11.8148	56.6741	28
Denmark	Lysen (12)	8.84003	56.6964	118
Denmark	Lysen (offshore) (12)	8.84003	56.7098	53
Denmark	Hals (42)	10.2941	56.9882	62
Denmark	Wadden Sea (129)	8.60196	55.2354	132
Sweden	Tjärnö	11.1527	58.8595	48
Netherlands	Eastern Scheldt	3.94953	51.5786	48
Norway	Agder	8.14725	58.1291	18

Determination of size classes

We estimated arbitrary size classes for each location according to size frequencies (length) of shells at each site, assuming these represent different age cohorts. We decided to use size frequencies (and not weight, for example) given the problematic of multiple algae and stones attached to the shells that could bias weight measurements. For each site, size frequency distribution analysis resulted in different size cut-off (Table 1 in Appendix 4.1). At each site, we identified distinct size classes that ranged from classes 0 to 5 (class 0 is the youngest and 5 the oldest), using also further size information from annual shore surveys performed by DTU Aqua. Given that recruitment in these populations does not occur every year and growth in Pacific oysters can vary significantly according to population structure and local environment conditions, some size classes were absent or did not present a continuous sequence of age classes in some sites. In some locations, shells in between abundance peaks of the size frequency distributions that could be from more than one class were assembled two size categories (e.g. 1-2 or 2-3), as well as when we did not have enough samples to form a

given category of size (Table 1 in Appendix 4.1). A summary of the size class distribution after the cohort assessment is shown in Figure 4.1.1.

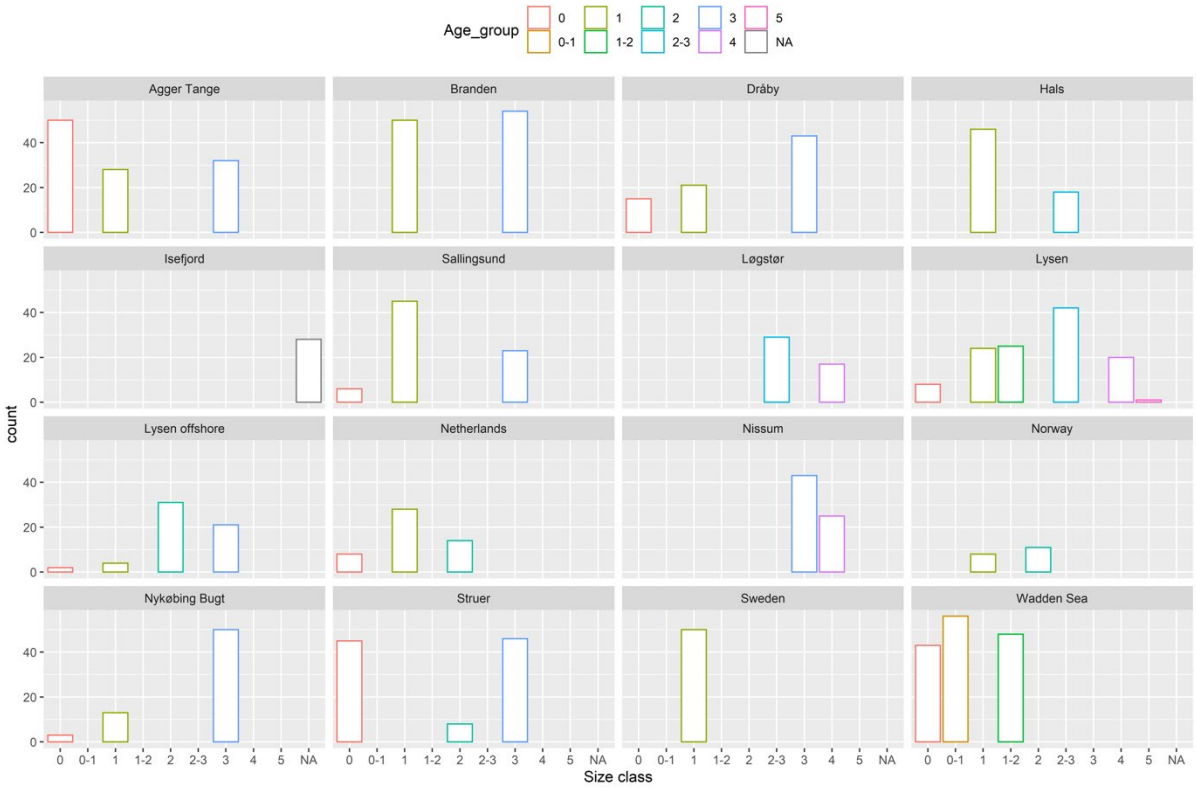

Figure 4.1.1. A summary of the distribution of size cohorts per location. For each shell sampled at a location, shell length was measured to generate size distributions used to determine size classes.

Development of SNP-chip

To investigate the genetic characteristics of the Pacific oyster populations in Denmark we use DNA information on different points in the genome, which we can collect through the genotyping of a SNP-chip, a method broadly used in genetics. In this project we developed a new SNP panel using previous knowledge of the genomic characterization of the oyster genome, in particular the previously described genome-wide SNP dataset by (Vendrami *et al.*, 2019). The Vendrami *et al.'s* SNP panel includes genetic information on many Pacific oyster populations around the world and it is therefore not specific to the populations in Denmark. By developing a new panel, we make sure that we get the best resolution in relation to the objectives of the project towards the genetic characterization of the Northern European population of Pacific oysters, and more specifically, to the Danish population. To create the SNP-panel, we selected the loci[3] that showed greater genetic differentiation between Denmark and UK, Sweden, Norway and Germany. In total a list of 116 SNPs was chosen for further testing on the SNP chip. As a quality test, we checked the SNP list generated to see the efficacy of the panel to differentiate between populations. For this test, we selected different individuals in two different training panels, with approximate 4-6 individuals per population in the training/test panel. SNPs that were not the same in both datasets were discarded. The final list of SNPs consisted of 115 SNPs. As a final check, genetic differentiation[4] (F_{ST}) for those SNPs were performed between Denmark,

[3] A general term to refer to a location in the DNA.

[4] Levels of genetic differentiation between two populations. E.g. F_{ST}=0, no differentiation; F_{ST} =1, two distinct isolated populations.

Sweden, Germany and UK, as well as a Principal Component Analysis, which is similar to a genetic map, to see if the signal observed remained consistent.

DNA extraction and SNP genotyping

To carry out genetic analysis on a given population, we extracted DNA from tissue samples. In the case of the Pacific oysters, DNA from all samples was extracted at DTU laboratory facilities from a piece of gill tissue using a Chelex DNA extraction method. We added 150 µl pre-heated (60 °C) 10% Chelex solution and 10 µl Proteinase K (20mg/ml), vortex every sample and incubated at 60 °C for 1 hour, and vortex every 20 minutes. Samples were then incubated during 15 minutes at 100 °C. After the incubation and once the samples reach room temperature, they were centrifuged 1 min at 13.000 rpm. All samples were run in 15 different Fluidigm runs using 96 SNPs from the 115 SNPs; each run contained 2 control individuals to check the replicability of the results. We used a Fluidigm IFC thermal cycler and BioMark instruments with SNPtypeTM chemistry for PCR amplification and genotyping, using 96.96 Dynamic Arrays. For calling genotypes, we used the BioMark Genotyping Analysis software (Fluidigm, San Francisco, California, USA).

Filtering and relatedness analysis

After the individuals are genotyped for the 96 SNPs from the SNP chip, we proceeded to filter the data to make sure that we take out any potential errors (e.g. laboratory process, machine-type errors). For example, genotypes were filtered for call rate under 80%, and missing data was also counted and filtered out individuals and loci with more than 30% of missing data. We also filtered out loci that had levels of linkage disequilibrium (measured by r^2) larger than 0.2, leaving a total of 62 SNPs for the rest of the analysis, out of the 96 SNPs that we started at the beginning. This filtering makes sure that the results are not due to any large missing data or other biases. We also measured the levels of relatedness within the individuals of each location using a specific software, COANCESTRY (Wang, 2011) with the Lynch & Ritland estimator. In parallel, to calculate the expected relatedness levels under random mating, we also simulated a new generation of 1000 individuals per population, using HybridLab (Nielsen et al., 2006). Finally, we compared the percentage of cases in both real and simulated datasets with a relatedness estimation above 4 standard deviations from the mean in the simulated dataset distribution, to check whether the real dataset follows the same expectations than a random-mating population.

Analysis of population structure and levels of connectivity

We measured the amount of genetic variation in the studied populations, using several measures of genetic diversity. In particular, we measured the observed Heterozygosity[5] (H_e), the 'allelic richness', i.e. the average number of variants per marker (may vary between 1 and 2) and F_{IS}[6], i.e. levels of inbreeding (see Glossary in Appendix 4.1). We mapped the SNP sequences from the remaining SNPs to the latest Pacific oyster genome (Peñaloza *et al.*, 2021) to characterize their position within the genome. We calculated F_{ST} per population, which is as a measure of population differentiation together with the p-values, which we Bonferroni-corrected. If the populations are genetically similar, levels of F_{ST} are 0; if the populations are genetically completely distinct, the F_{ST} will be 1. We also performed a Principal Coordinate Analysis (PcoA), which is another way to look at the measure of genetic differentiation. We quantified the levels of connectivity and gene flow and estimated migration levels (i.e. gene flow) between locations, using the G_{st} estimate[7], including only migration levels above 0.15. Contemporary genetically effective population sizes[8] (N_e) were estimated for each sampling location,

[5] Proportion of heterozygous loci observed in the DNA of an individual, averaged per population.
[6] Inbreeding coefficient of individuals in relation to the subpopulation. High F_{IS}-values imply considerable degree of inbreeding.
[7] Measure of differentiation between population that is used to calculate relative migration between them.
[8] The number of individuals reproducing/genetically contributing to the next generation.

using the "linkage disequilibrium[9]"-based method (LD-method). This method provides a snapshot of the effective population size, i.e. the smaller the effective population size, the fewer individuals has been contributing their genetic material to the present population. For the estimation of N_e, we filtered out loci with a frequency lower than 0.05, and confidence intervals (CI) were calculated.

Analysis of local adaptation

To identify if there were populations of Pacific oysters that are showing local adaptation, we ran a BAYESCAN outlier analysis (Foll & Gaggiotti, 2008). In this kind of analysis, we try to identify markers that may be showing a higher genetic differentiation (F_{ST}) compared to the average F_{ST} of the rest of the markers. A marker that has a very high F_{ST} could have a high probability to be on a gene (or close by) that is involved in local adaptation. We analysed six different scenarios in relation to study if there were evidence of adaptive differences between: the populations within the Limfjorden area, compared to among the same populations with either Isefjord, Norway, Sweden, Wadden Sea and the Netherlands. We used a prior odd of 10 which sets a higher threshold to validate a SNP under outlier in this analysis. The DNA sequence of loci highlighted as outliers by BAYESCAN were compared to a database with gene information (NCBI) to potentially identify their biological significance at the gene level.

4.2 Results

Data filtering and relatedness analysis

To check for consistency of genotyping, the control individuals present on every chip were included in a Principal Component Analysis (PCA) to visually inspect the relative positions in the "genetic map" of similarity among and check that their positions fall close to identical in the map. Once checked, they were discarded for subsequent down-stream analysis. After the filtering for call rate, individual missing data and LD-correlation (r^2) of 0.2, a total of 1100 individuals genotyped were used for the relatedness analysis. A first preliminary analysis of relatedness of the real data revealed two pairs of individuals with a relatedness of 1, likely to be individuals that had been processed twice in the lab. After the removal of one of each pair, we repeated the relatedness analysis for both the real data and the simulated data. Mean relatedness of the real data ranged from -0.0088 (Lysen) to -0.0393 (Isefjord) (Figure 4.2.1A, Table 2 in Appendix 4.1), with SD ranging from 0.12-0.14. For the simulated data, the mean was -0.0010 (Figure 4.2.1B, Table 2 in Appendix 4.1). Confidence intervals (CI) of relatedness from both simulated and real data overlapped, although the CI of the simulated data expanded the ones from real data in some locations (Figure 4.2.1B, Table 2 in Appendix 4.1), thus we analysed the distributions for each dataset. The number of cases with a relatedness estimation above 4 standard deviations from the mean were 0.06% and 0.05% for the real and simulated data, respectively (Figure 4.2.1C, Table 2 in Appendix 4.1). The finding of low average relatedness at all sites, demonstrates that many spawning individuals have contributed to the settlement at each location, i.e. that the recruitment at each site was not only from a few families. In concert with the low levels of genetic differentiation, this also suggest high migration among sites, in the Limfjorden. We thus proceeded to follow with the subsequent analysis without any further filtering for related individuals, as there seemed to be no relatedness levels in the real data above what was expected under a random mating population scenario.

[9] Non-random association of two loci within the DNA, located or not close-by.

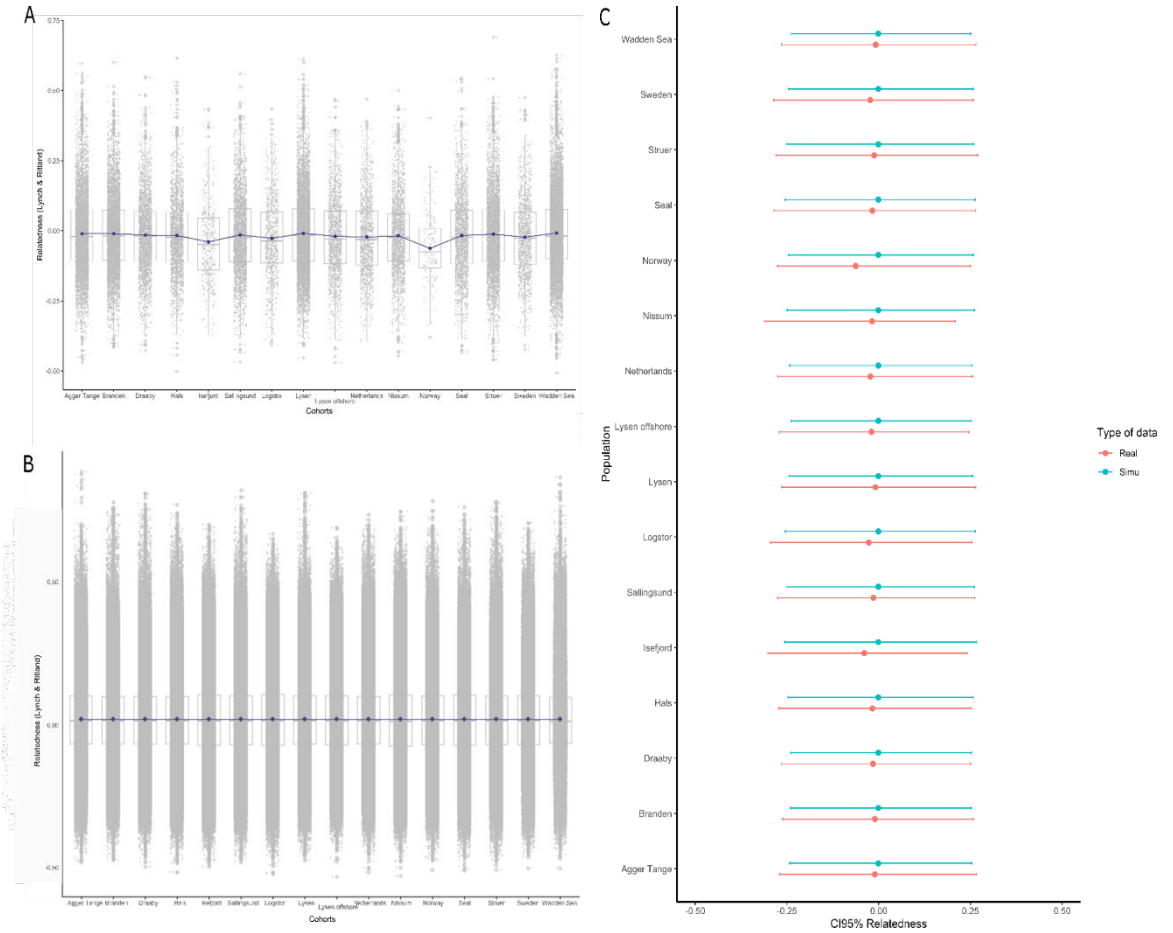

Figure 4.2.1. Relatedness analysis for the real (A) and simulated (B) data, and comparison of confidence intervals (CI) between real and simulated data (C).

A preliminary PCA analysis of the remaining individuals and loci showed still some patterns of remaining linkage within the SNPs (data not shown), driven by 4 SNPs identified by the loading plot of PCA1, which we proceeded to remove, leaving a total of 62 SNPs for the subsequent population genetic analysis. The average of missing data per population was 4.6%, ranging from 3% (Lysen Offshore) to 8% (Norway) (Table 4.2.1).

Table 4.2.1: Population genetic summary statistics. Comprising missing data levels and different population genetic statistics relative to the amount of genetic variation in the studied populations, described as Observed Heterozygosity (He), FIS, Mean Allelic Richness, and the effective population size (Ne).

Sampling site	Missing data	Observed H_e	F_{IS}	Mean Allelic Richness	No. of polymorphic SNPs	N_e (LD-method)	N_e CI 95%
Agger Tange	0.059	0.2453	0.1399	1.7895	4	894.4	243-∞
Branden	0.0334	0.2432	0.0971	1.7931	2	1499.6	274.8-∞
Dråby	0.0377	0.2435	0.0784	1.7903	1	884.4	167.5-∞
Hals	0.0372	0.2459	0.0816	1.7570	4	∞	204.1-∞
Isefjord	0.0478	0.2367	0.0902	1.7190	11	501.8	58.2-∞
Sallingsund	0.0373	0.2416	0.1003	1.7447	7	380.3	139.0-∞
Løgstør	0.0488	0.2389	0.1285	1.7396	10	260	77.9-∞
Lysen	0.0379	0.2521	0.0883	1.7771	3	690.4	244.2-∞
Lysen Offshore	0.0310	0.2477	0.0836	1.7705	6	666.9	130.3-∞
Netherlands	0.0437	0.2241	0.1685	1.8103	5	1268.4	141.3-∞
Nissum	0.0465	0.2415	0.1136	1.7747	4	781	137.4-∞
Norway	0.0789	0.2336	0.1384	1.7903	11	∞	395.1-∞
Nykøbing Bugt	0.0339	0.2516	0.0785	1.7339	9	450.1	130.4-∞
Struer	0.0482	0.2428	0.1016	1.7403	6	2957.7	242.4-∞
Sweden	0.0662	0.2424	0.1080	1.7779	6	∞	893.1-∞
Wadden Sea	0.0461	0.2343	0.1090	1.77885	1	419.1	199.1-10520.2

Genetic characterization of the population of Pacific oysters in Denmark

The level of genetic diversity within populations was assessed using two metrics, heterozygosity and allelic richness. The mean observed heterozygosity among the Danish Limfjorden populations was 0.245 and ranged from 0.238 (Løgstør) to 0.252 (Lysen) (Table 4.2.1). The non-Danish locations had lower heterozygosity estimates (Sweden: 0.242; Norway, 0.234 and the Netherlands: 0.224). Among the Danish locations, allelic richness oscillated from 1.719 (Isefjord) to 1.792 (Branden), and the F_{IS} ranged from 0.078 (Dråby, Nykøbing Bugt) to 0.1399 (Agger Tange). Mean allelic richness ranged from 1.719 (Isefjord) to 1.790 (Dråby) or 1.793 (Branden) among the Danish locations. Overall, this suggests that most populations have similar levels of genetic diversity. The slight decrease in diversity for Isefjord could be due to more isolation and independent colonisation history, while the small drop for Norwegian and Swedish samples could be due to the fact, that these sites were the last to be colonised. The PCA of the filtered 62-SNP panel mostly showed a homogenized oyster population among all locations analysed for this study, with the Netherlands' group showing some patterns of differentiation from the rest (Figure 4.2.2).

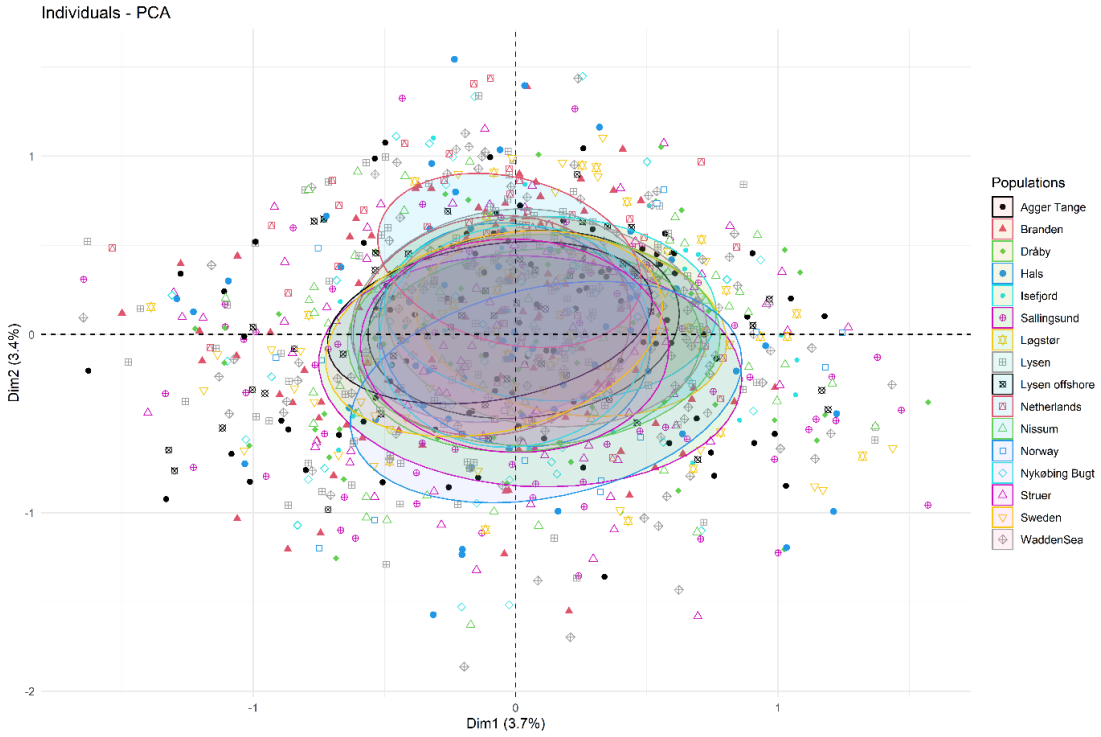

Figure 4.2.2. Genetic map of the relationship between individuals (Principal Component Analysis, PCA) included in this study, comprising the Limfjorden area (Agger Tange, Branden, Dråby, Hals, Sallingsund, Løgstør, Lysen, Nissum, Nykøbing Bugt and Struer), as well as the Wadden Sea, Isefjord area, the Netherlands, Sweden and Norway. Each point represents one of the 1100 individual samples analysed in the study. The ellipses represent the confidence intervals.

Out of the 96-SNP sequences from the full SNP-chip, 59 mapped to the latest Pacific oyster genome assembly (Peñaloza *et al.*, 2021), having at least 1 genomic hit in nine out of the ten linkage groups and in one scaffold (Figure 4.2.3), and from which 30 remained in the 62-SNPs that remained after filtering.

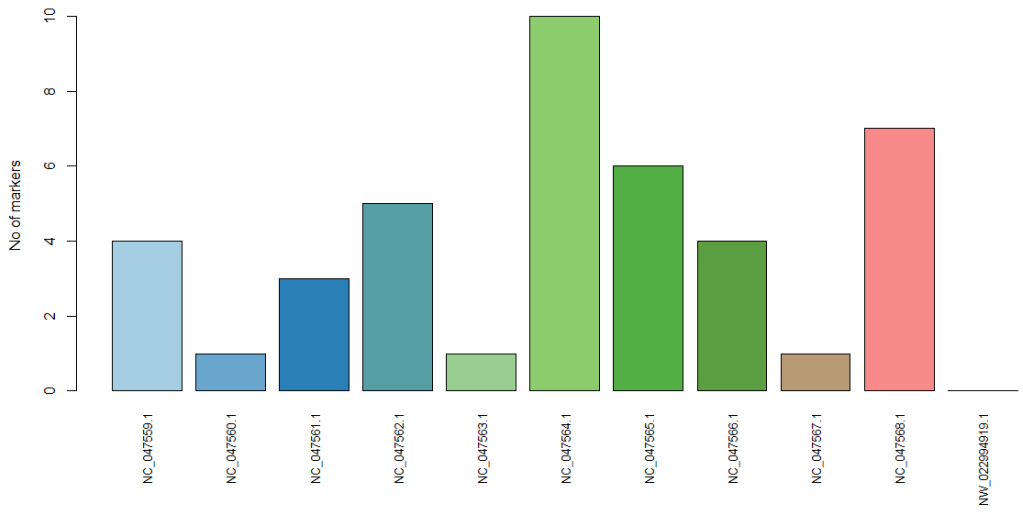

Figure 4.2.3. Number of SNPs from the SNP panel developed in this study, mapping to the Linkage Groups in the latest Pacific oyster genome assembly.

Among the samples located within the Limfjorden, F_{ST} values ranged from 0 to a maximum of 0.005 (Branden vs. Løgstør), with no statistically significant p-values after Bonferroni correction (Table 4.2.2). From the Danish populations, Isefjord presented the highest values of F_{ST} with the other Danish locations (between 0.009 with Struer, Nykøbing Bugt and Sallingsund, to 0.018 with Wadden Sea), with mostly significant p-values or slightly below the p-value threshold of 0.05. The Swedish and Norwegian samples presented highest F_{ST} values with the Danish populations from the Limfjorden area (between 0 to 0.006), although p-values were not statistically significant. Only the Isefjord and the Netherlands populations presented statistically significant F_{ST} values, which ranged between 0.01 and 0.03 (Table 4.2.2). The genetic differences of these two areas are also visualized in the PcoA (Figure 4.2.4), where one cluster represents the Danish Limfjorden populations, Sweden and Norway, separate from the Isefjord and the Netherlands samples. Based on the PcoA, the Wadden Sea sample appear to be genetically more closely associated with the Limfjorden samples than with the samples from Holland indicating a common colonisation history.

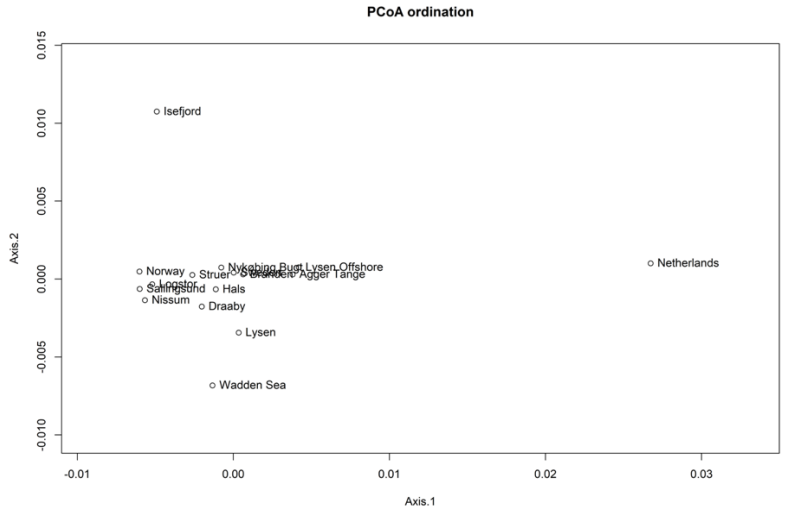

Figure 4.2.4. PCoA of the different sample locations.

The estimation of gene flow levels among populations (Figure 4.2.5) also showed this same pattern of F_{ST}. The mean effective population sizes of the Limfjorden populations were 946.48, with values ranging from a minimum of 260.0 (Løgstør) to an order of magnitude higher values, e.g. 2957.7 (Struer) (Table 4.2.1). Lower Confidence intervals (CI) of Ne ranged from 58.2 to 274.8 for the Danish populations, and all locations showed 95% CI that went up to infinity (Table 4.2.1). Levels of gene flow between locations (Figure 4.2.5) show values above 0.3 for all populations in the Limfjorden area and Sweden, all statistically significant. Norway also shares some gene flow levels with the Limfjorden area, but all lower than 0.3 (range: 0.16-0.21). In accordance with the F_{ST} and PCoA analysis, Isefjord and the Netherlands show lower gene flow with the Limfjorden area (0.16-0.20 and 0.09-0.15, respectively) (Figure 4.2.5).

Table 4.2.2: F_{ST} values (upper diagonal) and the corresponding p-values (lower diagonal) between the different locations. P-values are shown after Bonferroni correction.

P-values\ F_{ST}	Agger Tange	Branden	Struer	Nyøb-ing Bugt	Sallingsund	Dråby	Nissum	Løgstør	Isefjord	Lysen Offshore	Hals	Lysen	Netherlands	Sweden	Wadden Sea	Norway
Agger Tange		-0.001	0.000	0.000	0.000	-0.001	-0.001	0.002	0.011	-0.001	-0.002	0.000	0.020	0.000	0.001	0.004
Branden	0.9070		0.002	0.002	0.002	0.002	0.003	0.005	0.010	-0.002	-0.001	0.002	0.025	0.005	0.003	0.005
Struer	0.6636	0.2345		0.000	-0.002	0.001	-0.001	-0.002	0.009	0.000	0.001	0.002	0.029	0.000	0.002	-0.001
Nykøbing Bugt	0.7477	0.3185	0.6483		0.001	0.002	0.003	0.001	0.009	-0.001	0.002	0.002	0.027	0.001	0.004	0.002
Sallingsund	0.6786	0.1631	0.9702	0.5905		-0.002	-0.002	-0.002	0.009	-0.001	-0.001	0.003	0.033	0.002	0.001	0.003
Dråby	0.8249	0.1886	0.5800	0.2965	0.9056		-0.002	-0.001	0.012	0.000	-0.001	0.003	0.029	0.001	0.000	0.005
Nissum	0.8581	0.1800	0.8571	0.3215	0.9702	0.9702		-0.003	0.010	0.000	-0.001	0.002	0.033	0.001	-0.001	0.006
Løgstør	0.4982	0.1333	0.8249	0.5391	0.9289	0.7794	0.9702		0.009	0.003	0.001	0.001	0.032	-0.001	0.002	0.003
Isefjord	0.0000	0.0157	0.0157	0.0578	0.0508	0.0060	0.0432	0.0771		0.010	0.011	0.015	0.033	0.009	0.018	0.010
Lysen Offshore	0.8249	0.9771	0.6921	0.8242	0.8571	0.7794	0.7052	0.3766	0.0432		-0.003	0.002	0.019	0.002	0.000	0.004
Hals	0.9780	0.9070	0.5800	0.4406	0.8167	0.8249	0.8781	0.6091	0.0157	0.9702		-0.002	0.027	0.003	0.000	0.003
Lysen	0.7905	0.1362	0.2965	0.1873	0.1631	0.1303	0.2843	0.6270	0.0060	0.3600	0.9702		0.026	0.002	0.004	0.004
Netherlands	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.026	0.028	0.033
Sweden	0.6921	0.0882	0.7147	0.5800	0.3824	0.5886	0.5905	0.7813	0.0863	0.4239	0.2613	0.3766	0.0000		0.0007	0.0015
Wadden Sea	0.4406	0.0786	0.2093	0.0863	0.6415	0.7052	0.9070	0.3860	0.0000	0.7052	0.7787	0.0060	0.0000	0.5886		0.0080
Norway	0.3915	0.2965	0.7794	0.6031	0.4412	0.2965	0.2965	0.5391	0.0882	0.3766	0.5355	0.3687	0.0000	0.6091	0.0863	

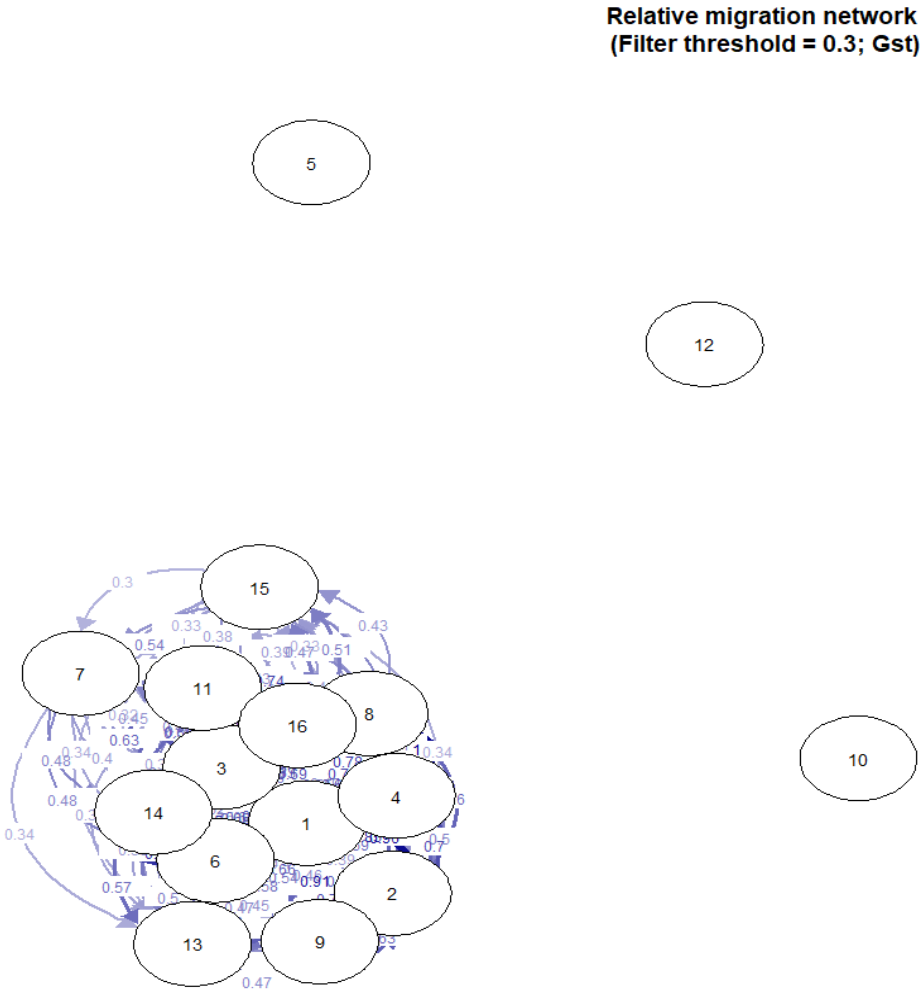

Figure 4.2.5. Relative migration network between the different locations analysed in this study. The relative migration network represents the relative gene flow levels between the different locations. Only values of migration above 0.3 are represented in the figure; all p-values between pairs of populations are significant. Legend: 1) Agger Tange; 2) Branden; 3) Dråby; 4) Hals; 5) Isefjord; 6: Sallingsund; 7) Løgstør; 8) Lysen; 9) Lysen Offshore; 10) Netherlands; 11) Nissum; 12) Norway; 13) Nykøbing Bugt; 14) Struer; 15) Sweden; 16) Wadden Sea.

Analysis of local adaptation

When running BAYESCAN, one SNP resulted as outlier in all scenarios tested ("AX-169162279") (Figure 4.2.6). This sequence where the SNP was found could be identified back to two possible regions in the M gigas genome, in Linkage Groups 02 and 06, however; without any information on the biological function of the gene or the area where this SNP is present.

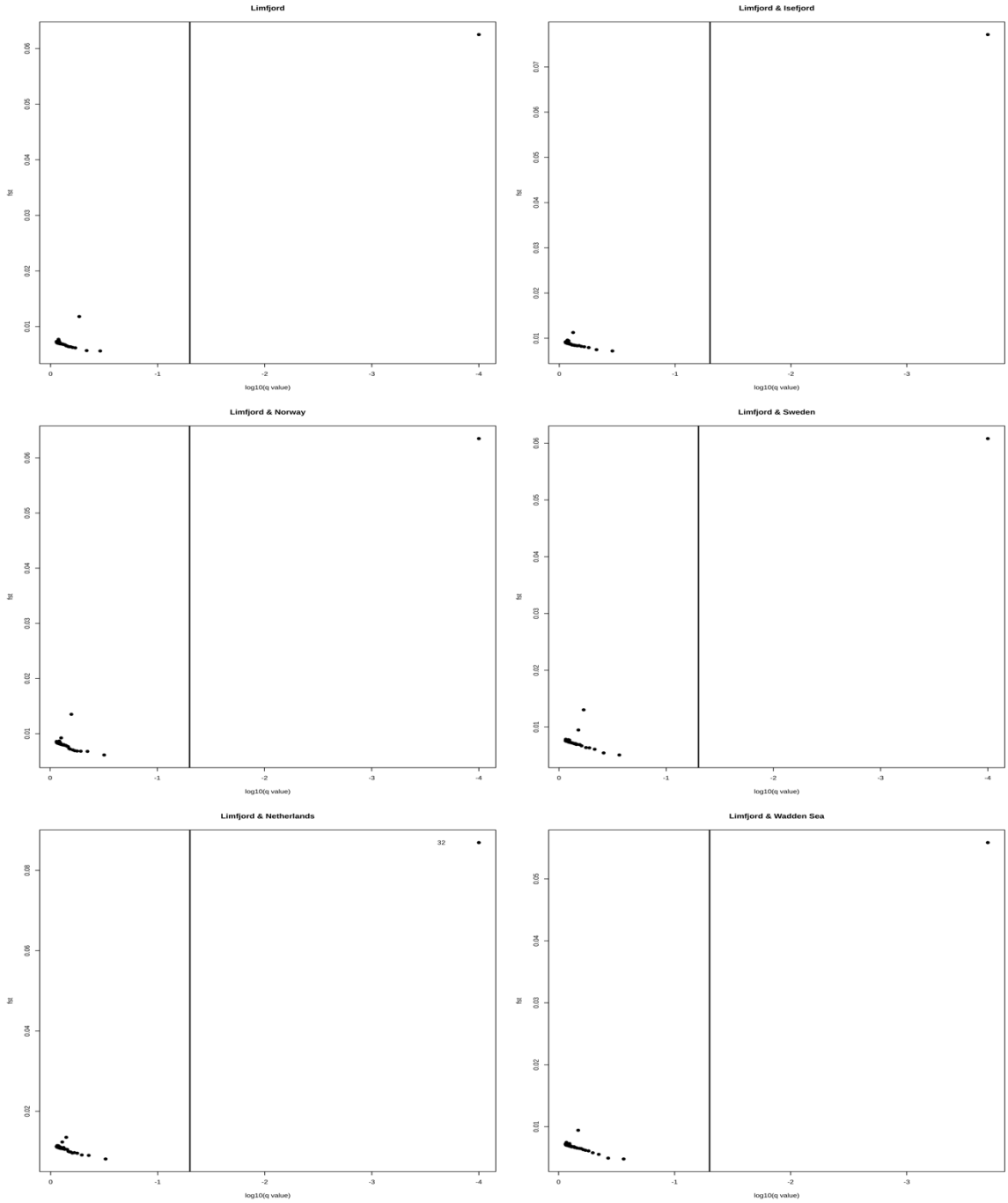

Figure 4.2.6. DNA markers that may be associated with genetic modifications within the oyster stocks. The comparisons have been made between: Limfjorden (all populations in the Limfjorden area); Limfjorden & Isefjord (all populations in Limfjorden plus Isefjord); Limfjorden & Norway (all populations in Limfjorden plus Norway); Limfjorden & Sweden (populations in Limfjorden plus Sweden); Limfjorden & Netherlands (populations in Limfjorden plus the Netherlands); Limfjorden & Wadden Sea (all populations in Limfjorden plus the Wadden Sea). Each figure for each scenario tested shows the degree of diversity between the 62 individual genetic markers (black dots) within the compared stocks. The F_{ST} on the y-axis of each figure shows the differentiation levels, and the x-axis shows the probability that the SNP is under different local selection (log 10 qvalue). The marker that appears to the right of the vertical line, with the highest degrees of genetic diversity, can potentially be associated with local genetic adaptations.

Discussion

We have analysed the genetic diversity and population structure in Danish waters (Limfjorden and Isefjord), using samples from Norway, Sweden and the Netherlands as outgroups.

Our results show no differentiation among the Pacific oyster populations within the Limfjorden area. Similar results were recently found for another bivalve species occurring in Limfjorden (blue mussel *Mytilus edulis*), where the genetic analysis of 23 SNPs did not show any genetic differentiation among the Limfjorden populations analysed (Kijewski et al, 2019; Pastor *et al.*, 2021). This means that the Pacific oysters in the Limfjorden do not possess genetic differences between local areas within the fjord, at least for the molecular markers that were analysed as part of this study and represent a single stock. In contrast, we found statistically significant differentiation between Limfjorden and Isefjord populations. These results follow the expectations of a larval drift simulation study using an agent-based dispersal model developed at DTU (Same-Risk-Area Assessment Model - Hansen & Christensen, 2018), where it was shown that 99% and 82% of larvae produced within Wadden Sea/Limfjorden area and Isefjord, respectively, settled within the same geographical area, with little to no connection between the two areas (F.T. Hansen, unpublished results). With respect to the non-Danish groups, the populations in Norway and Sweden did not show any statistically significant genetic differentiation with the Limfjorden or Isefjord populations. This result is also consistent with the study by Vendrami *et al.* (2019) who showed a clear clustering between the Northern and the Southern European populations, where the Northern samples included samples from Scandinavia and Germany. In contrast, the Netherlands showed differentiation with all the other populations. We observed the presence of high levels of gene flow within the Limfjorden populations, as well as populations in Sweden and Norway, which also follows expected patterns from agent-based dispersal models (F. T. Hansen, unpublished results). This means that larvae within the Limfjorden can settle more or less freely within the fjord. It has been previously suggested that populations in Sweden could have been a colonization from populations in Denmark (Rohfritsch *et al.*, 2013), which corroborates the high migration levels that we observe between Sweden and Limfjorden. Although our analysis has not focused on further studying the direction of gene flow, this could explain why we find no significant population differences in between the populations from the two areas.

Regarding the level of recruitment, the Pacific oysters within Danish waters present relatively large genetically effective population sizes (N_e), with no observed relatedness among individuals within the locations analysed in this study. Large effective genetic sizes means that recruitment is taking place involving many individuals rather than just a few, which also assures that genetic diversity can be maintained through time and is not rapidly lost in the short term. The N_e values that we observed for the populations in Denmark are within the CI ranges observed for the same species in other wild areas such as Ireland, where effective sizes have been estimated to be between 138.4 to 229.8, with a range of CI of 91.5-1121.2 (Kochmann *et al.*, 2012). We cannot discard that the genetic size of Pacific oysters could be larger than the estimates reported in this study, as it has been shown that Ne estimates can be downward biased when sample sizes are smaller when compared to the actual Ne (England *et al.*, 2006). Therefore, if more precise estimates are of interest, more individuals within locations should be included in the analysis to assess if this is the case for the populations analysed here. Our results have not shown patterns of local adaptation of the Pacific oysters in Danish waters, with respect to Pacific oysters from other non-Danish locations. The only outlier locus found as part of the BAYESCAN analysis did not reveal any particular gene under selection within the Pacific oyster genome, but we cannot discard that such marker may be hitchhiking with (linked) genes in the vicinity. Our study did not target the full genome of the oyster, so we cannot reject the possibility that there are regions of the genome under selection in other areas of the genome that we have not looked at in our study. To explore this, more analysis needs to be done involving a larger number of markers to cover

the whole genome or studying regions of the genome with protein coding genes which are anticipated to be the centres of genetically based local adaptations.

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5. Size-age relations in shallow Pacific oysters from the microtidal Limfjorden

Authors: Pedro S. Freitas, Camille Saurel, Charlotte Colvin, Ian McCarthy and Chris Richardson

5.1 Introduction

Pacific oyster populations have expanded significantly in recent years in shallow habitats in Danish coastal waters. In the microtidal conditions of the Limfjorden, Pacific oysters have often established dense and reef populations at depth s shallower than 1 m (Section 2).

Knowledge on the size at age relationships and growth of Pacific oysters under the shallow and micro-tidal conditions of Danish coastal waters is lacking and is important to assess the size-age distribution of wild populations and fully understand the invasion and expansion patterns of Pacific oyster populations in Denmark.

The aim of the study was to determine growth and size at age relationships in Pacific oysters from shallow habitats in the Limfjorden by following the size and growth of oysters over one year under a field experiment, using sclerochronological growth patterns (e.g. Richardson et al., 1999) and chemical elemental profiles (Durham et al., 2017; Haussman et al., 2017) in the shell to estimate age. Profiles of elemental concentration along the shell or hinge regions have been described as valid method to age oyster shells, as they may show annual cycles that result from forcing by annually varying environmental conditions, such as temperature (Durham et al., 2017; Haussman et al., 2017).

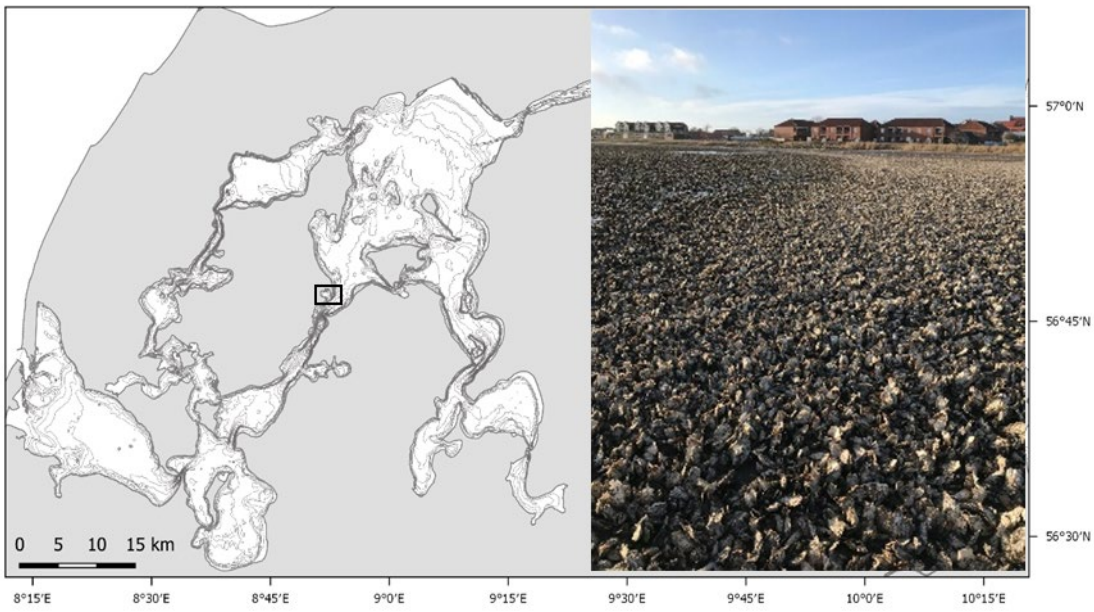

Figure 5.1.1. Location of trial site in Klosterbugten (black rectangle, right) on the Island of Mors in the Limfjorden, Denmark and photo of the exposed Pacific oyster bed (left).

5.2 Methods

Field experiment

Pacific oysters ranging in size from 40 mm to just over 200 mm in shell length were collected in October 2018 from a wild shallow population in Klosterbugten, Mors (Figure 5.1.1). Oysters were held for three weeks in tanks in the laboratory, labelled by drilling a number and gluing a label on the upper (right) valve. Oysters were placed in known compartments within plastic mesh cages attached to metal frames to keep it 30 cm above the bottom sediment (Figure 5.2.1). Cages were deployed for 12 months between November 2018 and November 2019 at ca.1.5 m depth in proximity of a shallow dense Pacific oyster bed near the DTU Aqua in Klosterbugten (Figure 5.1.1), which has been surveyed twice since 2018 (This project, unpublished). Cages were retrieved in April, May, July and September 2019 and on each occasion, cages were cleaned, mortality assessed and then returned to the field on the same day.

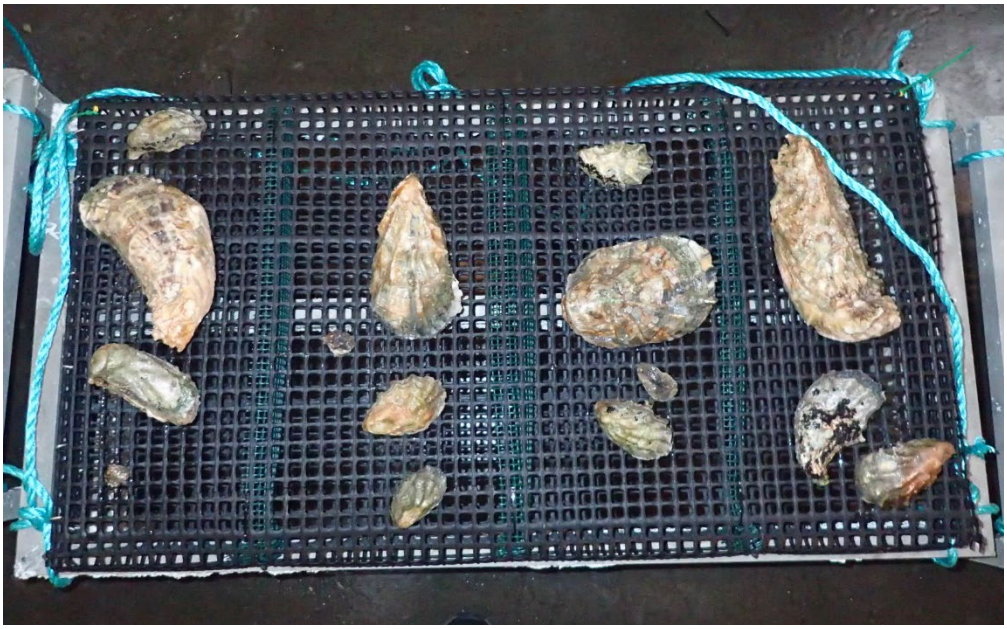

Figure 5.2.1. Cages with Pacific oysters before deployment.

Calcein marking

Oyster shells were marked twice with Calcein at the start of the experiment and six months later in May 2019 when evidence of new shell growth was observed. Calcein is a fluorescent dye that incorporates into growing calcified structures such as bone or shells often used as a growth marker in bivalve shells (e.g. Kaehler and MacQuaid, 1999; Mahé et al., 2010). The rationale of this approach was to place a mark in the shell at a given moment in time, allow the oyster to grow for a further 6 and 12 months and then section the shells to identify the Calcein mark and relate this to subsequent shell growth and to determine shell growth rates.

Marking was done by immersion for a period of 24h in tanks containing Calcein (Figure 5.2.2), at a concentration of 0.125-0.166 mg l^{-1} expected to result in 100% marking (Kaehler and MacQuaid, 1999; Mahé et al., 2010). Oysters were conditioned to the holding tank over a few days before marking with calcein, water temperature was kept at 11-12°C and a mix of microalgae was supplied. Oysters were observed to be open and feeding during marking. Oysters were collected at the end of the field experiment and dry shells were held in a light proof box to slow the processes of degradation of the fluorescent dye until processing

The whole of the shells of the Calcein-marked oysters were resin-embedded and sectioned as described below. Both the growing margin (where the Calcein dye would likely be detected) and the hinge region of the shells were analysed to investigate Calcein incorporation. Thin shell sections (approximately 0.2 mm thick so light could pass through the section) were prepared in the same way as sections were prepared for the LA-ICP-MS analyses above. Thin sections were observed microscopically at x10 in ultraviolet to investigate whether any fluorescent marks could be observed in the shells.

Figure 5.2.2. Calcein marking of Pacific oysters by immersion during 24h. Temperature was kept constant at 15 °C and oysters were fed microalgae.

Shell measurements

Oyster shells were measured (length and weight) and photographed at the start of the experiment in November 2018, in May 2019 and at the end of the experiment in November 2019. Each shell valve (n= 47) was imaged dorsally against a flat surface with a calibrated ruler and the image imported and analysed in ImageJ (Abramoff et al., 2004). Accurate measurements of the shell dimensions were undertaken (Figure 5.2.3). Total shell length (TSL) and umbo length (UL) were measured to the nearest 0.001 mm according to Richardson et al. (1993). The relationship between TSL and UL for the flat (upper) and concave (bottom) shells was described using linear regression analysis. In addition, shell width, the thickness of the shell, was measured to the nearest 0.1 mm in live shells with both valves attached.

The von Bertalanffy growth function was fitted to length and age data for the flat shell valve, which can then be used to model mean shell length from age: $Length_{(age)} = L\infty\ (1\text{-}e^{-K(age\text{-}t_0)})$, where age is age in years, L∞ is length at infinity when growth approaches 0, K is the growth coefficient and t_0 is theoretical age when size is 0. ϕ is the growth performance index (Pauly and Munro, 1984) = 2 * log($L\infty$) + log(K).

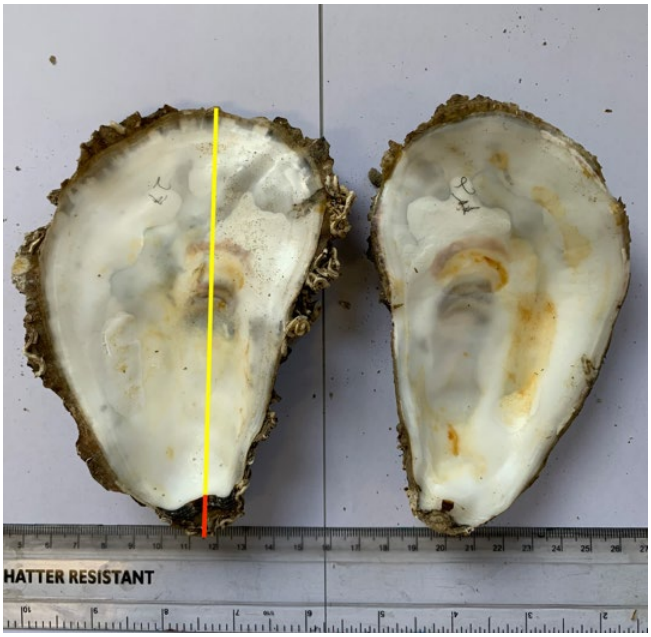

Figure 5.2.3. Dorsal view of Pacific oyster shells (concave shell on left and flat shell on the right) with ruler for length calibration. Yellow + Red line represents total shell length (TSL, mm), red line umbo length (UL, mm) measured using ImageJ.

Sclerochronological and geochemical measurements

Acetate peels

Acetate peels and LA-ICP-MS polished blocks were prepared using standard techniques. For acetate peels the block preparation was carried out using the methodologies outlined in Hollyman et al., 2020, and are summarised here. Oyster shells were embedded in MetPrep Kleer-set polyester casting resin and Blocks were cut with a diamond saw along the central point of the umbo region. Cut blocks were held flat and ground on a Struers rotation grinder on progressively finer silicon carbide grinding paper (p80-p4000). Once a flat surface was achieved, samples were fine polished using a 3-micron suspension on a Mecapol P230 rotation grinder until a clear surface was achieved.

One half of each polished umbo region was used to prepare acetate peels. Blocks were submerged in 0.1M HCL to be etched for 2 minutes. Blocks were then rinsed in water for 1 minute and air-dried before 2-3 drops of ethyl acetate were pipetted onto the surface. A cut square of acetate sheet (Agar Scientific replication material) was carefully placed over the top of the etched shell surface. Peels were left to dry for 30 minutes before removal, trimmed to remove excess, placed on a microscope slide and covered with a coverslip. Acetate peels were prepared for 39 Pacific oysters respectively. Samples were observed under a dissection microscope and aged by counting the number of growth lines in the umbo (Figure 5.2.4) as outlined in Richardson et al., (1993).

For LA-ICP-MS analysis, a block of <1 cm shell thickness was required to fit into the chamber of the laser. Following polishing of the shell blocks, the polished surface of selected blocks was attached to glass slides using superglue and the excess resin and shell material removed using a Buehler Isomet 5000 linear precision diamond saw to produce a thin slice of shell. The cut surface was ground and polished (as above).

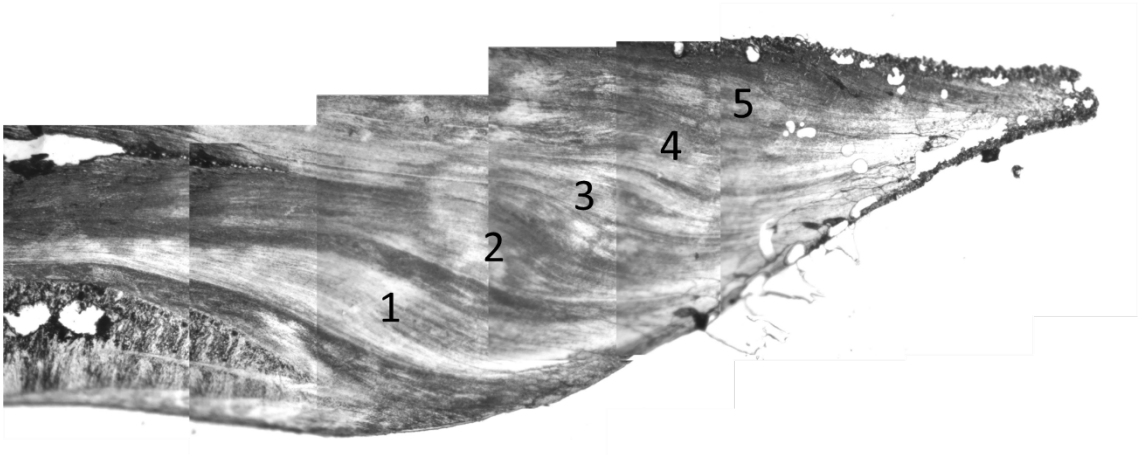

Figure 5.2.4. Microscope image at 10x magnification showing the annual growth lines on the acetate peel of the Pacific oyster (Sample P74; 5 years). Inner shell surface towards the bottom and umbo to top right.

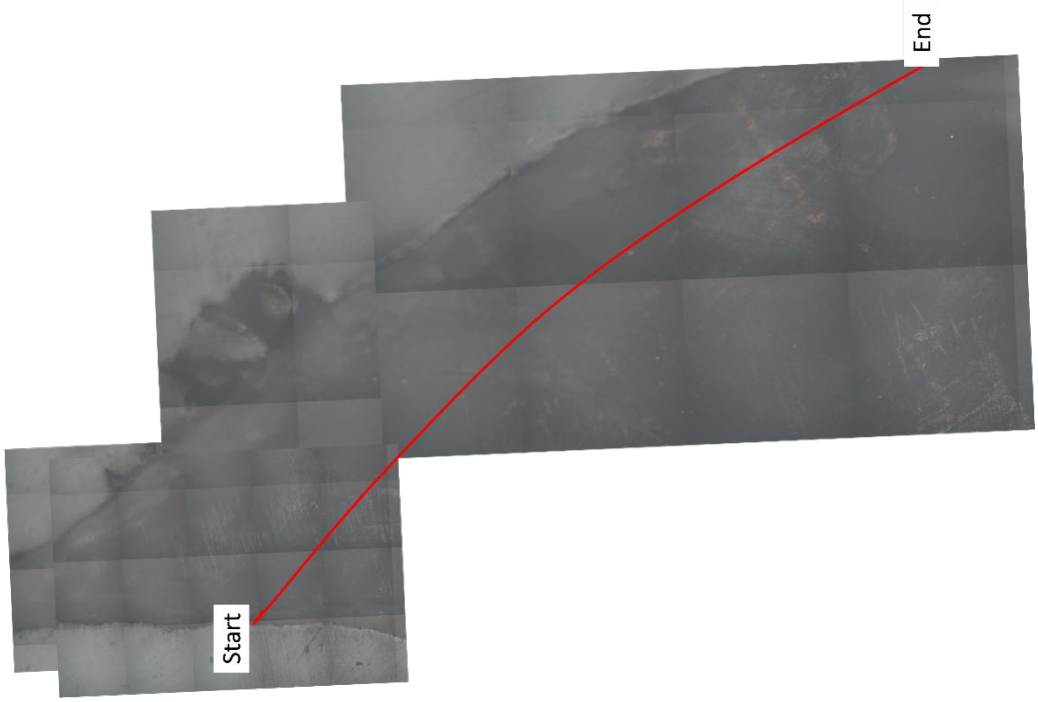

Figure 5.2.5. Map taken of the umbo region of shell (P74) under the LA-ICP-MS. The laser trace transect has been marked using the red line with start and end points shown. The tip of the umbo is to the left, the inner shell surface to the bottom and growth from left to right.

LA-ICP-MS

Thin sectioned blocks of ten Pacific oysters were analysed using an “imageGEO” laser ablation system (Elemental Scientific Lasers) coupled to an Agilent ICP-MS detector to assess variation in element concentrations across annual growth lines. A range of shells containing 2 to 9 growth lines (2 to 9 years old) were selected to run a series of laser spots across the umbo region. Two shells (5 and 6 years old) were further selected to run complete element maps of the entire shell section region. The elements Mg-24, Sr-88, Ba-134 and Fe-56 were measured. Ba-134 and Fe-56 showed no cyclical patterns across the transects and so are not presented here. The laser was run using a 17ms dwell time. 50-micron squares were used with a 5 second dwell and 2 second interval time across the 50-micron grid (and the inter-spot distance in the transect is 50 microns (Figure 5.2.5). Profiles of elemental composition along the shell or hinge regions have been described as valid method to age oyster shells (Durham et al., 2017; Haussman et al., 2017). Mg and Sr profiles are shown as Mg/Ca and

Sr/Ca ratios (ppm) and minima of Mg represent winter/cold season, with Sr the variation is noisier and less clear.

5.3 Results

Calcein Marking

Images of thin sectioned Calcein-marked oysters exposed to ultraviolet light were assessed for Calcein incorporation (Figure 5.3.1), however this proved unsuccessful, with no indication of Calcein related fluorescence under the UV light source. Although the periostracum (organic outer shell covering) of the sectioned shells did fluoresce, this may be due to the periostracum absorbing the Calcein dye during marking and/or dye absorption by particulates on the shell surface.

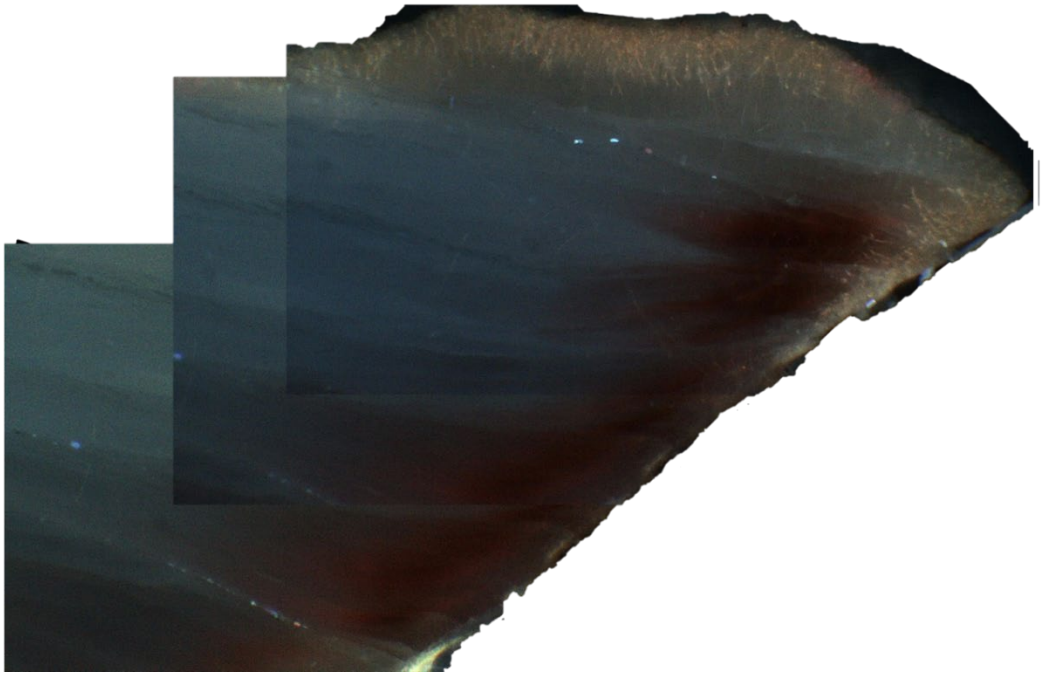

Figure 5.3.1. Representative composite image of a Calcein-stained oyster shell umbo indicating marking was unsuccessful and Calcein not incorporated. Areas of blue on the shell show fluorescence. Inner surface to bottom right, Umbo tip on the top right.

Length distribution

Frequency histograms were plotted for total shell length (mm) for both the concave (TSL) and flat (FSL) sides to the shell, as well as for shell width (mm) from live shells. TSL and FSL ranged between 40.3 and 228.1 mm and 35.7 and 229.3 mm, respectively, while shell width range between 31.3 and 110.5 (Figure 5.3.2). No clear presence of size cohorts is observed as sampling aimed at covering a wide range of sizes and potential ages.

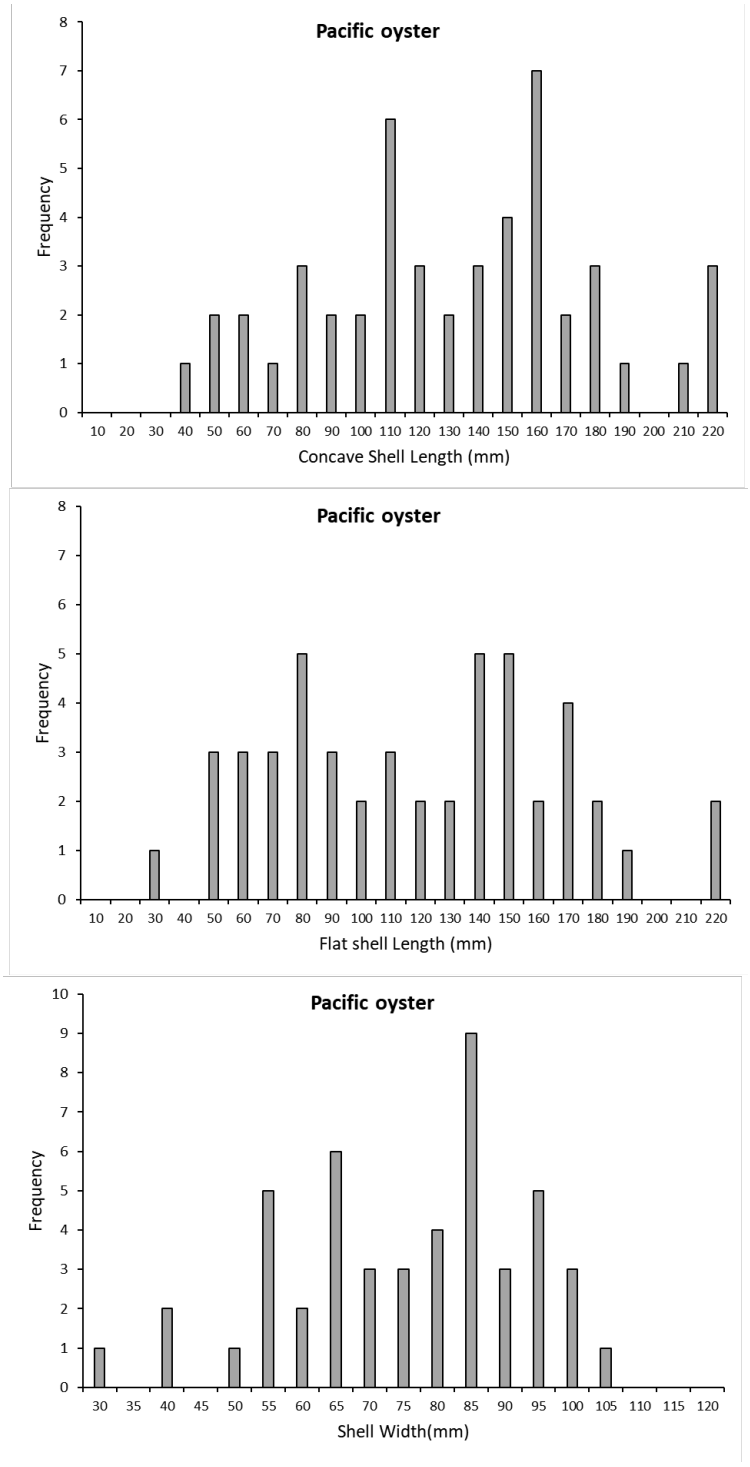

Figure 5.3.2. Total shell length (mm) frequency histograms for concave shell valve (top row) and flat shell valve (bottom row) of Pacific oysters *C. gigas* (N = 47).

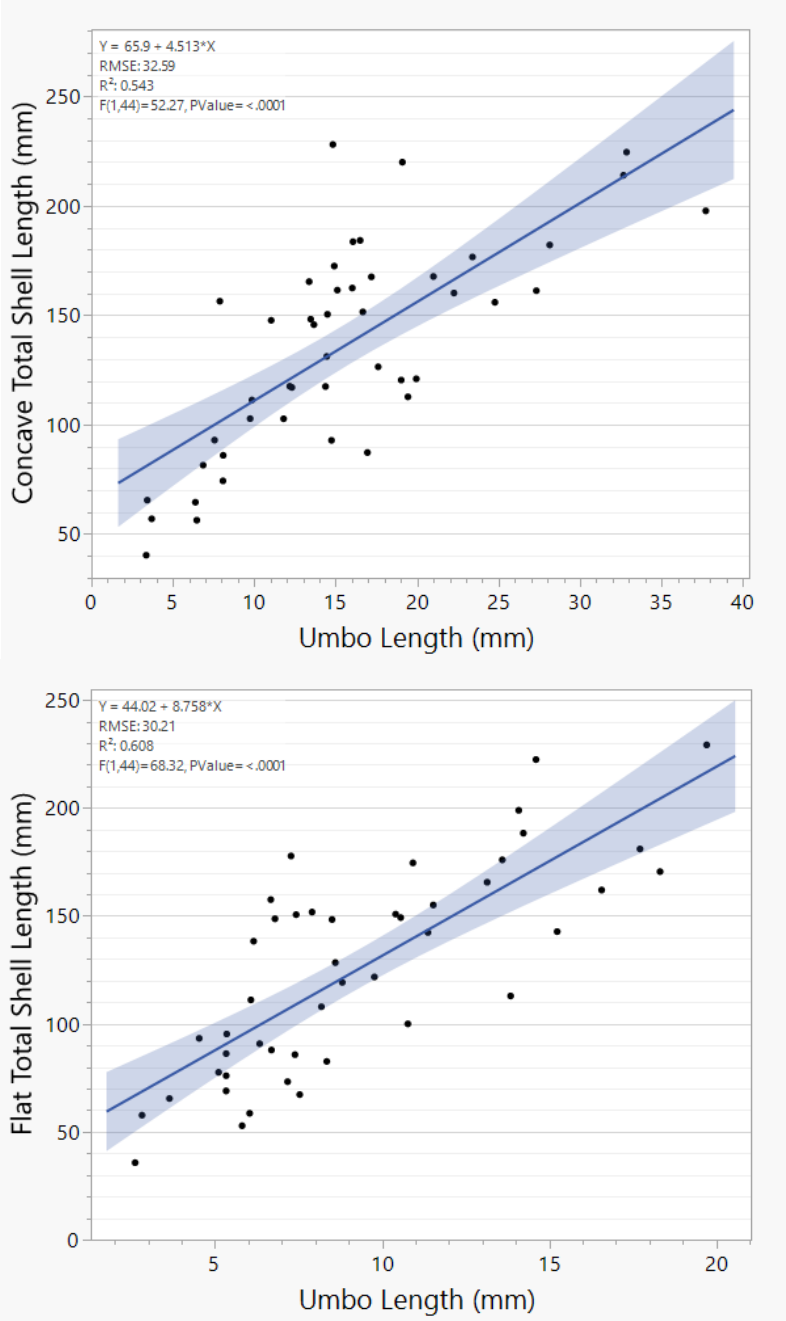

Figure 5.3.3. The relationships between umbo length (mm) and total shell length (mm) using the concave (top) and flat (bottom) shell sections for flat oyster O. edulis and Pacific oyster C. gigas. Linear regression equations are presented on each panel with 95% confidence limits of fit (shaded area), root mean square error (RMSE), coefficient of determination (r^2) and significance probability (F test, p value).

Total Shell/ Umbo Length Relationship

Figure 5.3.3 presents the relationships between umbo length (mm) and total shell length (mm) for the concave and flat shell valves for Pacific oysters. Both the flat and concave shell valves show significant positive linear relationships between umbo length and total shell length (all $p < 0.001$) providing calibration equations that can be used to estimate Total shell length from umbo length, for instance from shells that are dead and/or damaged. From the spread of data observed, and the goodness of fit (i.e. r^2, coefficient of determination) of the regression analysis, the flat valve is more suitable for predicting total shell length from umbo length (Figure 5.3.3). The rationale is that if measurements between the umbo hinge edge and each growth line are measured then using the linear relationships in

Figure 5.3.3, the total shell length of each oyster at a particular age (growth line) can be determined and thus shell size (growth) of each oyster can be revealed throughout ontogeny at each age. This was outside the scope of this study and was not undertaken.

Total Shell Length vs Age

The age range observed from growth lines in acetate peels of the umbo was 2-9 years for Pacific oysters (Figure 5.3.4). The relationship between shell size and age (i.e., growth plots) for Pacific oysters is shown in Figure 5.3.5. As expected, shell length increased with age, but the strength of the fit (r^2, coefficient of determination) was low at only 0.46. Table 5.3.1 presents the growth constants obtained by fitting the von Bertalanffy growth function to the length-at-age data using the flat shell valve data, which can then be used to model mean shell length from age.

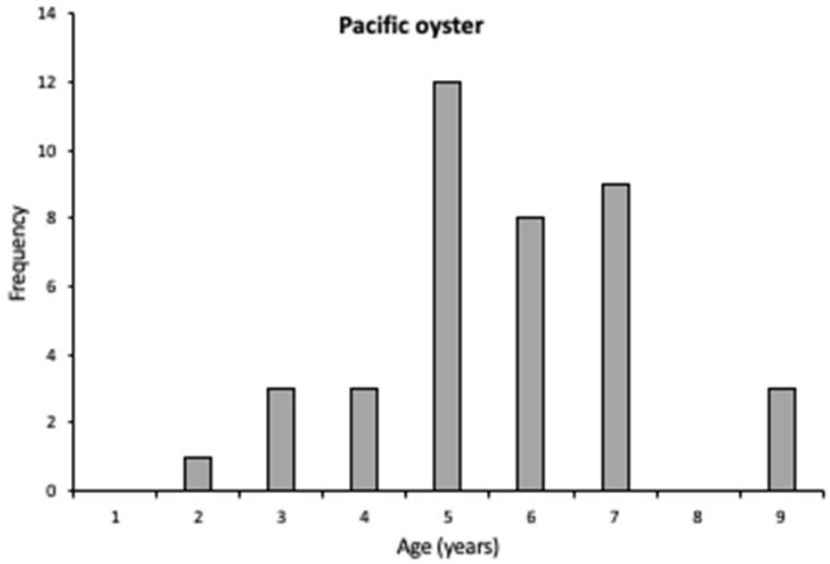

Figure 5.3.4. Age frequency distributions for Pacific oysters *C. gigas* (n = 47) from counting annual growth lines in the umbo regions of the flat valve of the shells.

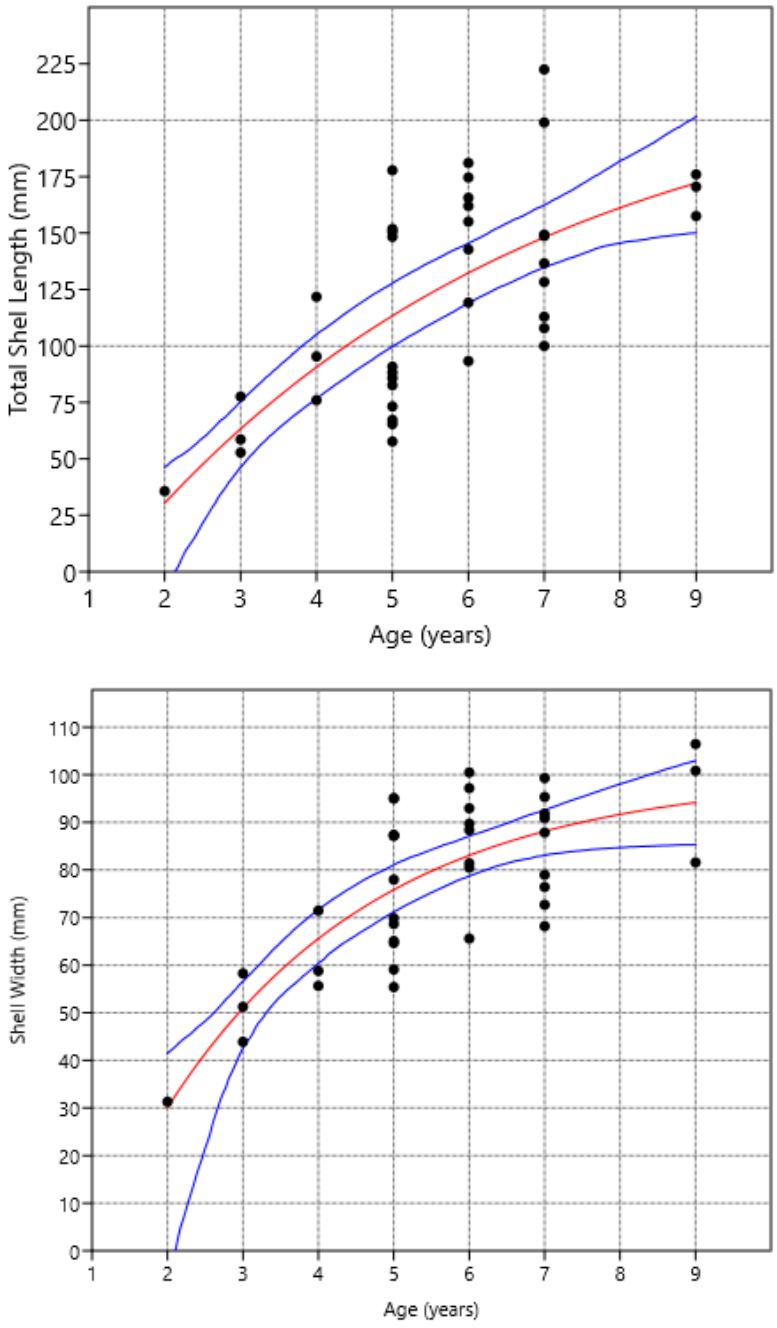

Figure 5.3.5. Relationship between flat shell length and age (top) and shell width and age (bottom) of Pacific oysters *C. gigas* (n = 39). Measures of TSL taken using flat shell side and shell width using live shells with both valves attached.

Table 5.3.1. Von Bertalanffy growth constants for Pacific oyster *C. gigas* using size based on the flat shell valve length (longest growth axis) as well as live shell width (thickness) measured with both valves attached.

	Flat shell length	Shell Width
N	39	39
L_∞ (mm)	226.3	100.1
K ($year^{-1}$)	0.184	0.354
t_o (year)	1.214	0.988
ϕ	3.974	3.549
r^2	0.456	0.588
p	0.001	0.001

Shell Mg and Sr composition

Analyses of the elemental composition of the umbo region were conducted along transects using a series of overlapping 50 µm laser spots (see Figure 5.2.5). The elemental composition profiles across the shell umbo region of the flat valve were undertaken to capture changes in Mg and Sr concentration occurring between and across the growth lines observed in acetate peels and shell sections (Figures 5.3.6 and 5.3.7).

Annual cycles in Mg and Sr concentrations could be observed in most shell transects, albeit not always clearly and with variable patterns between shells (Figure 5.3.6 and 5.3.7). Sr in particular, lacked clear and evident cycles in several shells (Figure 5.3.7). In 3 shells, Mg profiles could be interpreted has having a different number of cycles, while in Sr profiles, it was in 6 shells (Table 5.3.2).

Table 5.3.2. Comparison of ages obtained for 10 Pacific oysters *C. gigas* by counting the number of growth lines obtained in acetate peels and counting the number of cycles observed in Mg or Sr composition of the flat valve of the shell.

Shell No.	Number of growth lines	Number of Mg cycles	Number of Sr cycles
5.63	6	6	4 or 6
3.27	5	6	9 or 11
5.76	3	5	5
3.26	7	6 or 7	7
4.66	2 or 3	3	5
5.72	5	7	3 or 8
P74	5	5	6 or 7
P23	9	6 or 8	7 or 8
P85	2	1	1
P61	4	4 or 7	7 or 8

Agreement between the ages obtained using acetate peel growth lines and Mg and Sr annual cycles was generally poor (Table 5.3.2). The number of growth lines in peels was only equal at best to the number of Mg and Sr cycles in 50% and 20 % shells, respectively (Table 5.3.2). The number of growth lines in acetate peels under- or over-estimated the number of Mg and Sr cycles by one year in 30% and 30% shells, respectively and by two years or more in 20% and 50% shells, respectively (Table 5.3.2). Overall, the agreement to within ± one year was observed at best in 8/10 oysters when comparing acetate peels to Mg cycles and 5/10 when compared to Sr cycles.

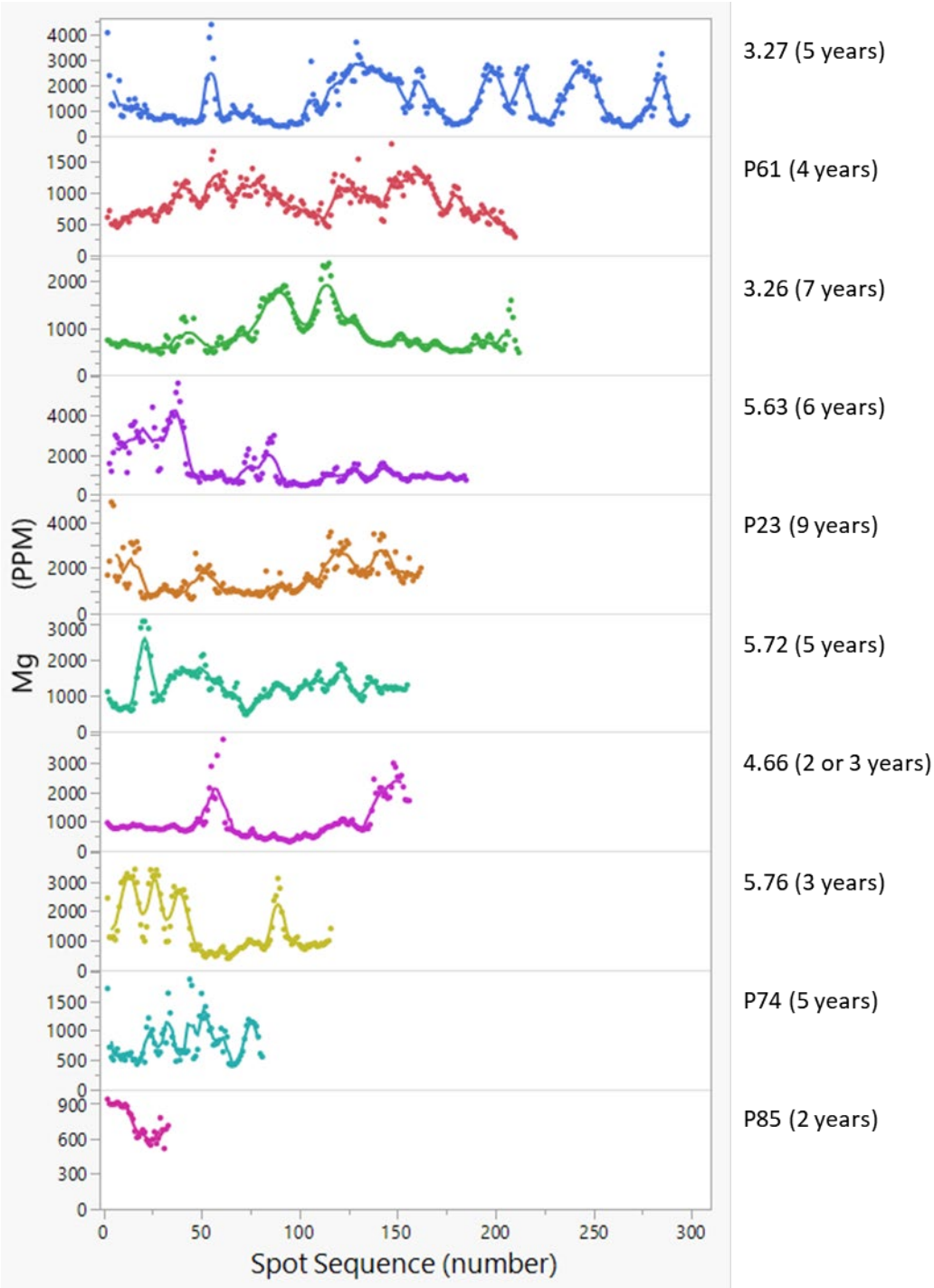

Figure 5.3.6. Transects through the umbo region of the flat valve of 10 Pacific oyster C. gigas showing changes in the Mg composition of the shell (ppm). Inter-spot distance in the transect is 50 microns and lines are moving average fits of variable width. The age of each oyster, determined by counting the number of growth lines in the shell is also presented on each plot. Note that some extreme outlying points are not visible on some plots as the y-axis scale has been set to allow the cyclical pattern in the majority of data to be visible. Cycles counted at Mg minima, which should correspond to the lowest annual temperatures.

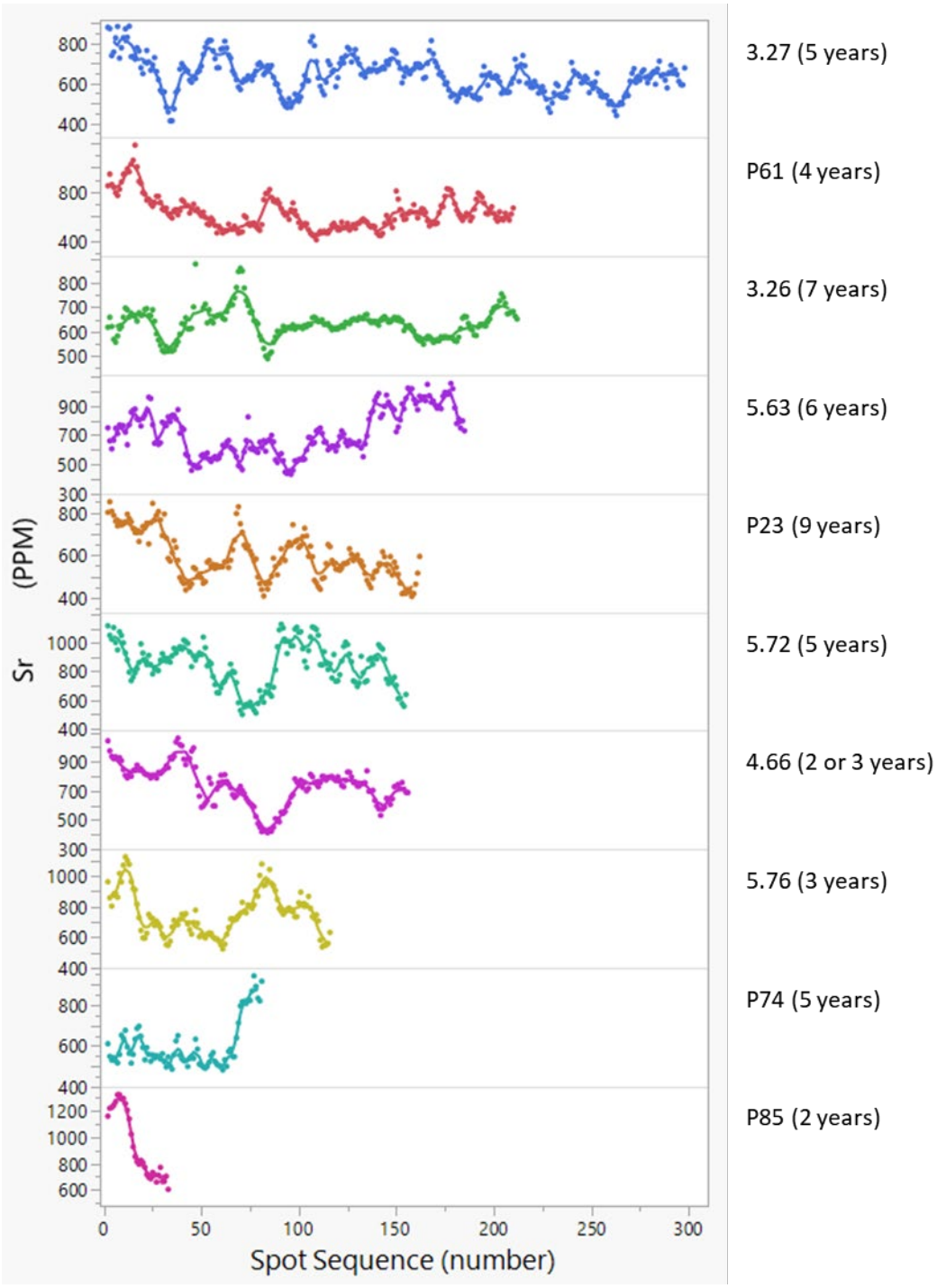

Figure 5.3.7. Transects through the umbo region of the flat valve of 10 Pacific oyster C. gigas showing changes in Sr composition of the shell (ppm) and the inter-spot distance in the transect is 50 microns. The age of each oyster, determined by counting the number of growth lines in the shell is also presented on each plot. Note that some extreme outlying points are not visible on some plots as the y-axis scale has been set to allow the cyclical pattern in the majority of data to be visible. Cycles counted at Sr maxima.

5.4 Discussion

This study produced a new assessment of growth and size at age for Pacific oysters under the conditions of the Limfjorden: very shallow, micro-tidal and variable environmental conditions, near its northern European distribution limit (Troost, 2010).

Pacific oyster shells were measured and aged ranging in size from 35 to 229 mm in length and in age from 2 to 9 years old. A significant relationship was established between total shell and umbo lengths that can be used to estimate total length of dead and damaged shells from umbo measurements or when applied to measurements of growth lines in the umbo to reconstruct shell size and growth of individual oysters at different ages.

Marking of Pacific oyster shells with Calcein to constrain growth during the field growth experiment, and thus constrain ageing and geochemical analysis of the last annual growth increment, proved unsuccessful. The likely explanation is the lack of incorporation of Calcein in the shell, even though new shell growth was observed at the time of marking and marking of bivalve shells by immersion has been shown to be effective in several species (Kaehler and MacQuaid, 1999; Mahé et al., 2010). However, Pacific oysters may require marking by injecting Calcein into the extrapallial space under better growth conditions for marking to be effective.

Determination of Pacific oyster age using growth lines/marks in acetate peels of the umbo of the flat valve proved feasible and reliable (Richardson et al., 1993). However, contrary to previous work in other oyster species (Durham et al., 2017; Haussman et al., 2017), the use of annual cyclic variations in shell Mg and Sr composition to age Pacific oysters from the Limfjorden proved unreliable. Shell Mg and Sr concentration and annual cycles showed significant inter- and intra-shell variation, which rendered ages obtained from shell Mg and Sr annual cycles unreliable when compared with umbo growth line ages. It is possible that the concave (left) valve may be more reliable and more appropriate for geochemical determination of oyster age (e.g. Durham et al., 2017), as its umbo is larger than that of the flat (right) valve and may preserve better growth and environmental variations. The latter was chosen to be analysed in this study, due to better fits between total and umbo lengths and also age.

The positioning of the laser transects may explain some of the observed discrepancies between the number of growth lines in peels and the number of Mg and Sr cycles, as the first or the last growth lines may have been missed or only partially sampled during geochemical analyses. Additionally, significant variation in element composition in oysters within growth increments, that is in parts of the shell with the same age (isochronous) can show different composition depending on their proximity to the hinge (Hausmann et al., 2019). However, counting cycles as Mg minima, corresponding to the lowest annual temperatures, minimizes such errors as both the youngest and oldest parts of the profiles should start in warm conditions during initial oyster post-settlement growth or at the end of the experiment (early autumn).

It is possible that highly variable environmental conditions in the extremely shallow areas of the Limfjorden significantly affect the Mg and Sr composition of Pacific oyster shells, disturbing preservation of annual cycles. Since the Pacific oysters used in this study come from very shallow locations (0.5 to 1.0 m depth), oysters experienced different environmental conditions during growth due to small changes in depth. In the Limfjorden, tidal amplitude is only 5-10 cm and water level vary in a non-cyclical way determined by short-term meteorological forcing, which can lead to strong changes in temperature, salinity or emersion. These results support rational that methods for ageing or environmental reconstructions based on the geochemical composition of bivalve shells, must be tested and validated for local conditions, especially under extreme or highly variable environmental conditions, and when near its distributional limits, such as in the Limfjorden for the Pacific oyster.

The strength of the size-age relationship obtained by fitting the von Bertalanffy growth function to the length-at-age was not strong compared to other studies on Pacific oysters (Harding and Mann, 2006;

Schmidt et al., 2008), although weak length-at-age relationships have been reported in inter-tidal locations in NW Europe (Cardoso et al., 2007). A possible explanation is the large phenotypical and morphological plasticity of Pacific oysters (e.g. Ernande et al., 2004), where shells can vary in shape and growth depending on population structure (e.g. in reefs or dense populations, in clumps or individually) and environmental conditions (e.g. food supply, depth, wave exposure, bottom substrate). Shell width provided a better fit length-at-age than shell length, possibly because of the three linear measurements of shell/body size it is the one that best reflects shell volume and thus may be less variable with growth and environmental conditions than shell length. Growth rates (K, 0.184 ad 0.354 year^{-1}) were low relative to other studies in inter-tidal conditions (K, 0.44 ad 0.73 year^{-1}, in Reed et al. (2020), possibly reflecting harsher conditions of such shallow habitats. However, overall growth performance (ϕ = 3.94 and 3.55*)*, which reflects growth rate and maximum length, was similar or higher than in inter-tidal areas of the North Sea (ϕ = 2.6 and 3.79, calculated from Cardoso et al. (2007 and Schmidt et al. (2008 in Reed et al. (2020)), but lower than in its native area (ϕ = 4.23, calculated from Harding and Mann (2006 in Reed et al. (2020)).

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6. Pathogen and disease screening of Pacific oysters from Danish waters

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Pacific oysters are known to carry several diseases (pathogens, e.g. virus, bacteria), parasites and other associated invasive species (Goulletquer et al. 2002), and it is therefore important to map and monitor the status of these pathogens as they might pose a risk not only for the Pacific oysters themselves but also for native bivalves in an area. With the increase in population size and further spread of Pacific oysters in Danish coastal waters, there is a higher risk of disease outbreaks that can affect commercial important bivalve species (blue mussels, flat oysters, and cockles). For instance, oyster herpes virus, which has previously almost exclusively affected Pacific oysters, has been reported abroad to spread to flat oysters (Lynch et al. 2010), and the Pacific oysters are also known to host the parasite *Bonamia*, which have caused high mortality of flat oysters across Europe since the 1970s (Laing et al. 2006). Wild populations of Pacific oysters in Denmark have not been monitored for any of the bivalve pathogens, and the status of the different pathogens in the wild populations of Pacific oysters are therefore unknown. In this study, Pacific oysters from Limfjorden, Isefjord and the Wadden Sea were screened for the pathogens Oyster Herpes Virus (OsHV-1) and the bacterium *Vibrio aestuarianus*, both known to be important disease causes in Pacific oysters, but also for the parasites *Bonamia ostreae* and *Marteilia refringens*, both being notifiable pathogens according to EU regulations and disease causes in flat oyster production as well as the latter in blue mussel production. *Bonamia ostreae* is causing disease in the native European flat oyster stocks in Limfjorden with the first finding in 2014, whereas *Marteilia refringens* until now never has been found in molluscs in Danish waters.

6.1 Materials and Methods

Preliminary processing of whole Pacific oysters for molecular analyses at DTU
Whole Pacific oysters were received in either fresh or frozen condition at the Section for Fish and Shellfish Diseases, DTU Aqua, in Kongens Lyngby. See Table 6.1.1 for overview of received samples. Sampling years ranged from 2016 to 2021, primarily focusing on the Limfjorden (fishery zones 1 to 33 and 216), but also with one sampling done in the Isefjord (zone 110) as well as samplings in the Wadden Sea (zone 129).

Fresh oysters
The Pacific oysters were cleaned on the outside of the shell to remove any excess dirt, silt, sand and algae before further processing. A standard oyster knife was used to open each oyster and the oyster flesh was transferred from the shell to a clean plastic cutting board. Shells were put in numbered plastic bags, left opened, and set to dry in a fume hood. They were later transferred to DTU Aqua in Nykøbing Mors for age determination. The heart of the oyster was removed for making heart imprints on glass slides, which were coloured for screening for *Bonamia*. Three cross section slices were cut from each oyster, one for histology transferred to Davidson fixative, one for DNA extraction of tissue, and an extra for keeping at -20 °C.

Frozen oysters
The Pacific oysters were left out on a clean lab bench for thawing. When thawed, a standard oyster knife was used to open each oyster and the oyster flesh was transferred from the shell to a clean plastic cutting board. Shells were put in numbered plastic bags, left opened, and set to dry in a fume

hood. Two cross section slices were cut from each oyster, one for DNA extraction of tissue, and an extra for keeping at -20 °C.

Molecular Characterization

Genomic DNA extraction from tissue

From each oyster cross section slice prepared for DNA extraction, a total of 25 (±5) mg of tissue was cut from a mix of gill-, mantle- and digestive tissues. The 25 (±5) mg sample was transferred to a 2 ml Eppendorf tube used for lysing.

Table 6.1.1. Overview of received samples of Pacific oysters from the Limfjorden, the Wadden Sea and the Isefjord, Denmark.

Sample Batch ID	Sampling Month	Sampling Year	Sampling Area	Sampling Zone	Sampling Site	Sampling Depth	Sampling Co-ordinates	Condition of Samples	Number of Samples
SOE001-16-A-01	September	2016	Limfjorden	1	Lemvig	-	-	Alive	18
SOE001-17-01	January	2017	Limfjorden	1	Lemvig	-	-	Alive	29
SOE002-19-01	March	2019	Limfjorden	2	Nissum Bredning	4-6 m	56° 39.358' N 08° 19.171' E	Alive	90
SOE012-21-01	July	2021	Limfjorden	12	Lysen Bredning	0-2 m	56° 41.211' N 08° 50.422' E	Alive	90
SOE013-17-01	January	2017	Limfjorden	13	(DSC) Anlæg Sallingsund 61G	5-9 m	56° 47.099' N 08° 54.903' E	Alive	40
SOE013-17-02	November	2017	Limfjorden	13	(DSC) Anlæg Sallingsund 61G	5-9 m	56° 47.099' N 08° 54.903' E	Alive	40
SOE013-20-01×	March	2020	Limfjorden	15	Klosterbugten	0-2 m	56° 47.487' N 08° 51.646' E	Dead	10
SOE013-21-01	August	2021	Limfjorden	13	Klosterbugten	0-2 m	56° 47.487' N 08° 51.646' E	Alive	90
SOE015-21-01	August	2021	Limfjorden	15	Branden	0-2 m	56° 47.851' N 09° 01.579' E	Alive	90
SOE033-19-01	March	2019	Limfjorden	33	Løgstør	7-9 m	56° 55.297' N 08° 59.297' E	Alive	50
SOE216-19-01	April	2019	Limfjorden	216	Agger Tange	0-2 m	56° 41.257' N 08° 15.416' E	Alive	33
SOE110-19-01	November	2019	Isefjord	110	Søminestationen + Munk-holmbroen	0-2 m		Alive	28
SOE129-18-01	May	2018	Wadden Sea	129	Ho Bugt	0-2 m		Dead - frozen	30
SOE129-18-02	May	2018	Wadden Sea	129	Ho Bugt	0-2 m		Dead - frozen	38
SOE129-18-03*	May	2018	Wadden Sea	129	Ho Bugt	0-2 m		Dead - frozen	31
SOE129-18-04*	May	2018	Wadden Sea	129	Ho Bugt	0-2 m		Dead - frozen	35

× Misnamed SOE15-20-1as it was sampled in zone 13
* Very small in size < 5 cm

The QIAmp® DNA Mini Kit (QIAGEN®, Cat. No. 51304/51306) was used for DNA extraction of all samples, and the manufacturer's protocol was followed. The method only deviated from the manufacturer's protocol for two steps. For the first lysing step, samples were left at 56 °C overnight (up to 14 hours) and for the elution step, elution was done with 130 µl of Buffer AE and with a 5 min incubation before centrifugation.

DNA quality and quantity for each sample were measured by optic density at 260 nm using a NanoDrop™ 2000 Spectrophotometer from Thermo Fisher Scientific. All samples were diluted to appropriate DNA concentrations for respective PCR investigations. All DNA extractions were stored at -20 °C when not in use.

Quantitative Real Time Polymerase Chain Reactions
All qPCR investigations were performed using an Mx3005P qPCR system from Agilent Technologies®. All qPCR methods used for disease screenings were developed and published by the European Union Reference Laboratory for Mollusc Diseases, Ifremer, Laboratoire de Génétique et Pathologie, La Tremblade, France.

Marteilia refringens & *Bonamia* sp. or *Vibrio aestuarianus* screening
For screening the oysters for the protistan parasites *Marteilia refringens* and *Bonamia* sp. at the same time, a duplicate qPCR protocol was used developed by Ifremer (Canier et al. 2020, Link1). To screen the oysters for the gram-negative bacterium *Vibrio aestuarianus* another qPCR protocol, developed by Ifremer, was used (Garcia et al. 2021, Garnier et al. 2008, Nhung et al.2007, Saulnier et al 2009, Tison & Seidler 1983, Link2). Both qPCR protocols were followed without deviations except for the testing of different DNA sample concentrations. The qPCR mix for both protocols was prepared using either 2X Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies®, Cat. No. 600880) or 2X Luna® Universal Probe qPCR Master Mix (New England Biolabs, Cat. No. M3004S, M3004L). The two master mix products have proven to give comparative results.

Ostreid Herpesvirus type 1 (OsHV-1) screening
To screen the oysters for Ostreid Herpesvirus type 1 a published protocol from Ifremer (Pépin et al. 2008, Segarra et al. 2010, Webb et al. 2007, Link3) was once again followed except for testing various DNA concentrations. The qPCR mix for this protocol was prepared using 2X QuantiTect™ SYBR® Green PCR Master Mix (Qiagen®, Cat. No. 204141). This master mix has proven to give similar results as the one used in the referenced protocol.

Result evaluation criteria
Threshold Cycle (Ct) is defined as the cycle, where a statistically significant rise in fluorescence output above background levels is detected.

Controls
Before evaluating and concluding on the results of the tested samples, Ct-values of controls were checked. Negative controls (control samples where nuclease free water was added instead of DNA) should not present any amplification and thereby have no Ct-values. Positive controls (control samples known to be positive of the tested pathogen(s)) should present significant amplification within an established Ct-spectrum. If all controls presented the expected Ct-values or lack of same, the qPCR run was deemed successful, and an evaluation of the tested samples could commence.
Samples
All positive samples should present a sigmoidal/logarithmic curve and all negative samples should present a linear curve. All tested samples were checked to see if they represented either. A tested

sample was considered positive if its Ct-value was below 35. If samples presented a sigmoidal/logarithmic curve and had a Ct-value between 35 and 40, they were considered weakly positive. Samples with a Ct-value between 40 and 45 were considered negative if they presented a linear curve. If these samples presented a sigmoidal/logarithmic curve, they were deemed suspicious, but could be evaluated as very weakly positive if the curve was approved. Samples with Ct-values above 45 were considered negative.

6.2 Results and discussion

Overall, the molecular disease screening of Pacific oysters in the Limfjorden, the Wadden Sea, and the Isefjord found very low levels of pathogens present. An overview of the results can be seen in Table 6.2.1. Most of the oysters were found to be without any of the four pathogens chosen for the screening. Only 28 oysters were collected from the Isefjord, with all of them being from very shallow waters and only in November. None of these 28 oysters was found to carry any of the four chosen pathogens. This might be due to the low water temperatures normally found in November in shallow waters of the Isefjord (estimated 8-9 °C) (DMI, free data), as this is known to be unfavorable for the tested pathogens (Arzul et al. 2009, Kerr et al. 2018, Lupo et al. 2019, 2020, Parizadeh et al. 2018, Petton et al. 2013, Richez et al. 2012). Furthermore, the low number of oysters is not enough to evaluate the overall health status of Pacific oysters in the Isefjord. In general, no pathogens were found in oysters collected in colder months of the year, which correlates with most disease and mortality being observed in Pacific oysters in Europe during warmer summer months (Alfaro et al. 2019, EFSA 2010, Nguyen et al. 2019).

Bonamia sp. have never been observed to cause disease in Pacific oysters (Culloty et al. 1999), but it has previously been shown that Pacific oysters are able to carry *Bonamia* sp. (Helmer et al. 2020, Lynch et al. 2010), wherefore it was relevant to test the Pacific oysters in this study for the presence of *Bonamia* sp. The screened Pacific oysters did not test positive for the pathogen although *Bonamia ostreae* has been known to be present in the Limfjorden since 2014 (Madsen & Thomassen 2015). The lack of *M. refringens* in the screened Pacific oysters correlates with past testing, as this protistan parasite has not been found in any tested molluscs species from Danish waters so far.

Only two individuals were found positive for OsHV-1 of all Pacific oysters screened, although, one of these samples was within the weakly positive range, indicating a low infection level. The two positive oysters were found among a total of 134 (1.5%) oysters collected in Ho Bugt in the Wadden Sea on the 24th of May 2018 at low tide in an area, where water levels at high tide reaches no more than 2 m. May 2018 was exceptionally warm, dry and sunny compared to the norm, beating many old weather records. The average air temperature for that specific month was 15°C for the whole country with more than 15 days with average temperatures above 25°C, which equals 4.2°C above the norm for the month of May in Denmark. It also had a high number of sunny hours (505) compared to the norm 364 hours (DMI, free data). Especially the end of the month where the oysters were collected contributed substantially to the record breakings, and the average sea temperature for Esbjerg Harbour, very close to Ho Bugt, was 16.2 °C for the day the oysters were collected (DMI, free data). The very warm conditions at the end of the month mimicking summer temperatures would probably be the most likely reason for the findings of OsHV-1 in two of the collected oysters. Especially as the shallow water in the bay will be highly affected by the high number of sunny hours and high temperatures. An increase or sudden change in water temperature has been shown to be a high risk factor in the virulence of OsHV-1 (Clegg et al. 2014, EFSA 2010, Garcia et al. 2011), which this sudden heat wave in May

would have produced. Unfortunately, no oysters were collected from the same area later in the season to check for a possible progression of the pathogen (potentially leading to disease and mortality) during the summer months. Still, these results show that OsHV-1 is present in Ho Bugt and potentially other places in the Wadden Sea, and they indicate that the virus could become an issue for Pacific oysters in the area, if mean sea water temperatures continue to rise and sudden temperature changes continue to be observed more often (DMI).

Four of the sixteen batches of Pacific oysters screened had individuals testing positive for the bacterium *V. aestuarianus*. The location of the samplings of these four batches is shown in Figure 6.2.1.

Table 6.2.1. Overview of disease positive animals per month, year and water depth for Pacific oysters in Denmark.

Sample Batch ID	Sampling Month	Sampling Year	Sampling Depth	Total Samples	*Marteilia refringens* positives	*Bonamia sp.* positives	*Vibrio aestuarianus* positives	OsHV-1 positives
Limfjorden								
SOE001-16-A-01	September	2016	-	18	0	0	0	0
SOE001-17-01	January	2017	-	29	0	0	0	0
SOE002-19-01	March	2019	4-6 m	90	0	0	0	0
SOE012-21-01	July	2021	0-2 m	90	0	0	18[2]	0
SOE013-17-01	January	2017	5-9 m	40	0	0	0	0
SOE013-17-02	November	2017	5-9 m	40	0	0	2[1]	0
SOE013-20-01ˣ	March	2020	0-2 m	10	0	0	0	0
SOE013-21-01	August	2021	0-2 m	90	0	0	6[3]	0
SOE015-21-01	August	2021	0-2 m	90	0	0	13[4]	0
SOE033-19-01	March	2019	7-9 m	50	0	0	0	0
SOE216-19-01	April	2019	0-2 m	33	0	0	0	0
Isefjord								
SOE110-19-01	November	2019	0-2 m	28	0	0	0	0
Wadden Sea								
SOE129-18-01	May	2018	0-2 m	30	0	0	0	2[5]
SOE129-18-02	May	2018	0-2 m	38	0	0	0	0
SOE129-18-03*	May	2018	0-2 m	31	0	0	0	0
SOE129-18-04*	May	2018	0-2 m	35	0	0	0	0

ˣ Misnamed SOE15-20-1, as it was sampled in zone 13
* Very small in size < 5 cm

[1] Very weakly positive
[2] Positive to weakly positive
[3] Very weakly positive
[4] Weakly positive to very weakly positive
[5] Positive to weakly positive

Three of the four batches, SOE12-21-1 with 18 positive individuals of 90 total (20 %), SOE13-21-1 with 6 positive individuals of 90 total (6.7 %), and SOE15-21-1 with 13 positive individuals of 90 total (14.4 %), were all collected in the peak of summer in 2021 in mid-July and the end of August. The last batch, SOE13-17-2, with only 2 positive individuals of 40 total (5 %), were collected in late November 2017, but both individuals were very weakly positive. The SOE12-21-1 batch of oysters had the highest rate of *V. aestuarianus* positive individuals and the highest level of detection with individuals ranging from positive to weakly positive. SOE13-21-1 had only very few positive individuals that were all very weakly positive, and SOE15-21-1 had a few, less positive individuals than SOE12-21-1, but a lower detection level with individuals ranging from weakly positive to very weakly positive.

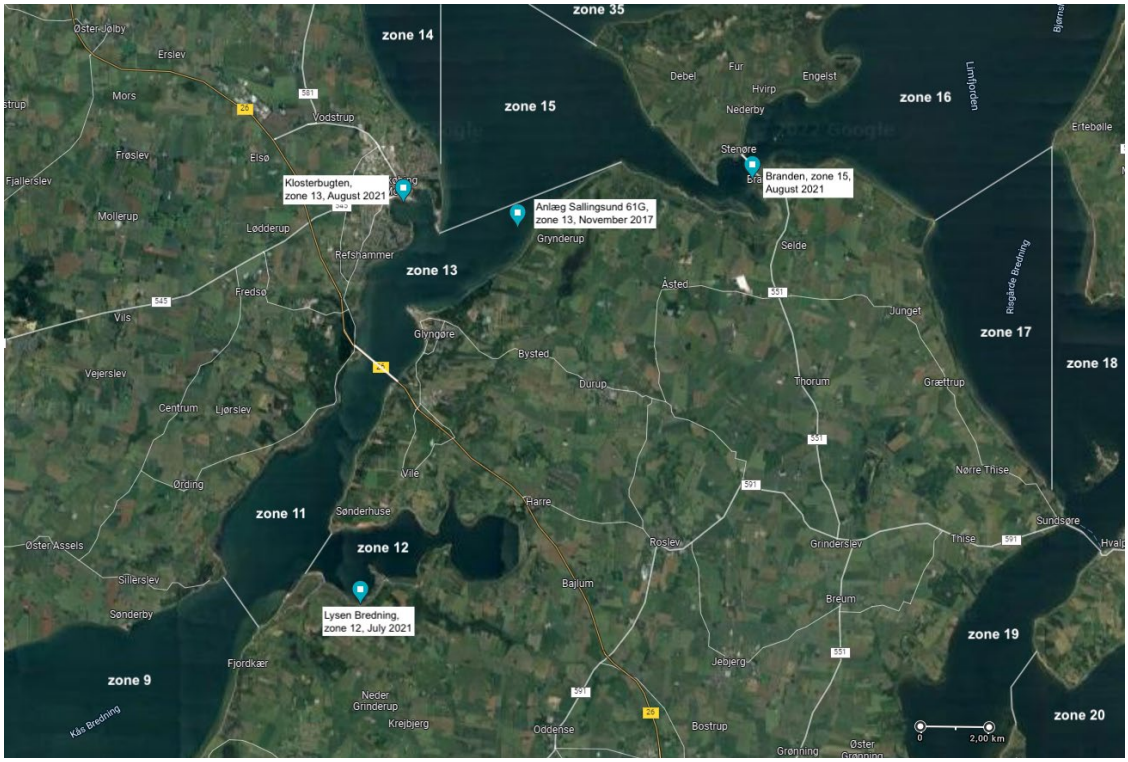

Figure 6.2.1. Map of the four locations where samples of Pacific oysters from the Limfjorden, Denmark were found positive of *Vibrio aestuarianus* in November 2017, July 2021, and August 2021.

Although *V. aestuarianus* was detected in three different fishing zones with locations being within a maximum distance of 17 km from each other, all positive individuals had a low to very low-grade infection. None of the oysters found positive of *V. aestuarianus* showed clear signs of disease and looked overall healthy when dissected, consistent with a very low level of infection. The results indicate that even in the peak of Danish summer in very shallow water in the Limfjorden, where estimated water temperatures would have been between 17 and 25°C (DMI, free data), Pacific oysters seem to deal well with *V. aestuarianus*. This would be despite water temperatures reaching 19°C and above have proven a major risk factor of Pacific oyster mortalities by pathogens if colliding with other stress factors, such as a high reproductive effort, eutrophication, and pollutants from freshwater discharges, decrease in dissolved oxygen, other pathogens or viruses, etc. (Lupo 2019, 2020, Travers et al. 2017). As the disease-causing pathway for *V. aestuarianus* is not completely elucidated, one explanation for the low levels of infection of *V. aestuarianus* might be that not enough other stress factors were present at the time of sampling (Alfaro et al. 2019, EFSA 2010, Lupo 2019, 2020, Petton et al. 2013).

No bacterial isolations on agar plates were done during the study, and the PCR detection protocol for *V. aestuarianus* does not discriminate between virulent and non-virulent strains of the bacterium. Therefore, it is not known if the *V. aestuarianus* detected in the screened oysters were virulent strains or not. Therefore, another reason for the low-grade infections found might be because the *V. aestuarianus* detected during the screening were a non-virulent strains (Nhung et al. 2007, Travers et al. 2017).

Overall, no high levels of infection by any of the pathogens screened for were found in the molecular screening of Pacific oysters from various Danish waters sampled at different time points of the year. Both OsHV-1 and *V. aestuarianus* were found at very low levels in some of the screened oysters proving their presence in the Wadden Sea and Limfjorden. If average water temperatures in those two areas continue to rise, these two pathogens might, especially in unity and together with increasing population densities of Pacific oysters as well as higher periodical rainfall, give rise to summer mortality events in Pacific oysters, as already observed in France, the United Kingdom, and the Republic of Ireland (Alfaro et al. 2019, EFSA 2010, Lupo 2019, 2020, Nguyen et al. 2019, Parizadeh et al. 2018, Petton et al. 2013, Richez et al. 2013).

6.3 References

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LINKS:

Link 1: https://www.eurl-mollusc.eu/content/download/118237/file/M.refringens%26BonamiaSp%20_RealTimePCR_editionN%C2%B01.pdf

Link 2: https://www.eurl-mollusc.eu/content/download/151132/file/Vaestuarianus%20_RealTimePCR_editionN%C2%B04.pdf

Link 3: https://www.eurl-mollusc.eu/content/download/42545/file/OsHV-1%20RTPCR_1.pdf

7. Experiences and identified challenges in developing sustainable fisheries of Pacific oysters

Authors: Pernille Nielsen

7.1 Experiences and identification of challenges with current fishery permits for Pacific oyster

Since 2015, there have been a growing interest in commercial utilisation of the invasive Pacific oyster in both the Wadden Sea and Limfjorden and several fishing licences have been given but with varying successes. In 2014, the first licence was given in the Wadden Sea to a larger commercial fishing vessel. However, the fishery was not economically viable due to few individuals of market sized oysters and the catches were dominated by large oysters of uneven sizes and clumps of oysters. Since the middle of the 2010s, Pacific oysters have been caught as by-catch either in the flat oyster or blue mussel fisheries at water depths >3 m in the Limfjorden. Like in the Wadden Sea, the Pacific oysters often do not have market size or shape but since they are caught as by-catch there is no extra costs for the fishers to catch them. Within the last 3-5 years, economically viable commercial fisheries of Pacific oysters carried out by hand collection or with hand-held tools have originated in the Limfjorden in the shallow areas (<3 m) and in the Wadden Sea.

Licences and landings of Pacific oyster in the Limfjorden and Wadden Sea 2017-2021
Currently in the Limfjorden, the Pacific oysters is either caught as by-catch in the blue mussel and flat oyster dredge fisheries at >3 m depth or collected by hand in the shallow areas <3 m. However, in 2017, 16 vessels applied for licences for a targeted fishery of Pacific oysters and a total of 4.4 t was landed by three vessels (Table 7.1.1). In 2018, two vessels had a licence but did not land any Pacific oysters and in 2019 one vessel landed 4.7 t. In 2020 and 2021 five vessels landed 2.7 and 14.6 t of Pacific oysters as by-catch, respectively. Of the five licences, one (2020) and two (2021) of the fishers also collected Pacific oysters by hand. The commercial hand collection of pacific oysters started in 2017, where one licence landed 4.6 t, which equals the landings in the targeted dredge fishery (Table 7.1.1). Like the targeted dredge fishery, no landings were reported for the permitted three licences for hand collection in 2018 but increased to 7.1 t in 2017 (two out of three licences active) and >9 t in 2020 and 2021, where all (two and three, respectably) licences were active. Of the 16 vessels getting a licence in 2017, only two of the vessels were landing Pacific oysters in 2021 and furthermore also started hand collection in 2021.

Table 7.1.1. Total number (no.) of licences, active licences in brackets and reported landings (tons) for either dredge fishery or hand collection of Pacific oysters in the Limfjorden and the Wadden Sea from 2017 to 2021. Data provided by Fiskeristyrelsen.

Limfjorden	2017	2018	2019	2020	2021
No. licences dredge fishery (active)	16 (3)	2 (0)	1 (1)	5 (5)	5 (5)
Landings dredge fishery (t)	4.4	0.0	4.7	2.7	14.6
No. licences hand collection (active)	6 (2)	3 (0)	3 (2)	2 (2)	3 (3)
Landings hand collection (t)	4.6	0.0	7.1	9.7	9.5
Total landings (t)	9.0	0.0	11.8	16.5	24.0
Wadden Sea	**2017**	**2018**	**2019**	**2020**	**2021**
No. licences hand collection (active)	0	0	2 (1)	2 (1)	2 (1)
Landings hand collection (t)	0	0	2.1	2.6	1.8

For hand collection, one licence has landed oysters every year since 2019, whereas another has either landed oysters from hand collection or dredge fishery. This indicates that there has been a flexibility among the few dedicated fishermen fishing for Pacific oysters in the Limfjorden and that the fishery in recent years appears to be profitable (Table 7.1.2).

Table 7.1.2. Maximum, minimum, and average price (DKK per kg) for live Pacific oyster caught in the Limfjorden by dredge fishery (bycatch) in 2017-2021, hand collection in 2020-2021 and hand collection in the Wadden Sea in 2021. Data provided by Fiskeristyrelsen.

	Bycatch from dredge fishery					Hand collection	
Limfjorden	**2017**	**2018**	**2019**	**2020**	**2021**	**2020**	**2021**
Max (DKK per kg)	2.0	8.0	8.0	5.0	15.0	35.0	35.0
Min (DKK per kg)	2.0	2.0	1.0	1.5	5.0	10.0	15.0
Average (DKK per kg)	2.0	3.5	3.0	2.5	9.1	19.0	16.3
Wadden Sea				**2020**	**2021**	**2020**	**2021**
Max (DKK per kg)	-	-	-	-	-	-	56.0
Min (DKK per kg)	-	-	-	-	-	-	25.0
Average (DKK per kg)	-	-	-	-	-	-	27.1

The price for hand collected Pacific oysters from the Limfjorden was at least twice the price of the dredge oysters, whereas the prices for the hand collected oysters in the Wadden Sea were approximately twice the price for the hand collected Pacific oysters in the Limfjorden (Table 7.1.2). Based on the information from the stakeholder feedback interviews of the fishers (see details below) the hand collected Pacific oysters are easily sold either to local traders in the Limfjorden area or at the auctions in Hanstholm or Thyborøn. The smallest sized Pacific oysters (80-120 g) give the highest prices (10-20 DKK per kg) when sold to local traders, whereas the prices at the auction were 3-5 DKK per kg.

In the Wadden Sea, two licenses for hand collection of Pacific oysters have been permitted each year since 2019 but only one licence per year have been active. The landings vary between 1.8 to 2.6 t per year (Table 7.1.1) with an average price of 27.1 DKK per kilo in 2021 (Table 7.1.2). Generally, the size of the hand collected Pacific oysters vary between 75 to 200 g and with an average price of 10-15 DKK per oyster (Source: feedback stakeholder interviews).

Bycatch of Pacific oysters in Natura 2000 sites in the Limfjorden 2017-2020

The population of Pacific oysters in the deeper parts (>3 m) of the Limfjorden is often within Natura 2000 areas. In Denmark, bivalve fisheries within Natura 2000 sites are not only limited by quotas but also by a maximum of 15% cumulative areal impact, which is assessed by implementation of the electronic monitoring system Black box (further details see Nielsen et al. 2021). Consequently, the focus within the Natura 2000 sites have been on more profitable blue mussel and flat oyster fisheries, where Pacific oysters were caught as bycatch and not in a targeted fishery, as this potentially could limit the other more profitable fisheries if the upper limit of 15% cumulative areal impact was reached. Consequently, the fishers have suggested that the areal impact form a targeted Pacific oyster fishery within Natura 2000 sites should not be accounted as 100%, as they are mitigating the effects of an invasive species.

To assess the areal impact caused by the bycatch of Pacific oysters in the flat oyster fishery, black box data combined with the landings from the two Natura 2000 sites Løgstør Bredning and Nissum Bredning in Limfjorden from 2017 to 2020 were used to estimate the densities of the Pacific oysters, landings per track and the areal impact per kilo bycatch of Pacific oyster. The results are presented in Table 7.1.3 and show relatively large variation in the area impacted between years within the same

Natura 2000 site, but also between the two areas. The variations in the area impacted is most likely caused by the change in flat oyster densities, as the Pacific oysters were caught as bycatch in the flat oyster fishery. From 2017 to 2019, the population of flat oysters in Nissum Bredning was reduced by half and the population of flat oysters in Løgstør Bredning was reduced by one third from 2019 to 2020. The decrease in the flat oyster population was likely due to increased mortality caused by the parasite *Bonamia.* The results clearly indicate, that the area impacted per kilo of bycatch Pacific oysters will vary both between the years and the areas and cannot be used to estimate a typical average areal impact would be in a targeted Pacific oyster fishery, as the areal impact most likely is reflecting the large variations in the flat oyster population rather than differences in densities of Pacific oysters in the two Natura 2000 sites, Nissum Bredning and Løgstør Bredning.

Table 7.1.3. Average values of bycatch densities (kg m^{-2}) of Pacific oyster (PO), average landings per track and the average area impacted per kg PO bycatch fished in Nissum Bredning and Løgstør Bredning from 2017 to 2020. The assessments are based on black box data combined with reported landings.

Nissum Bredning	2017	2018	2019	2020
Average density of PO (kg m^{-2})	0.002	-	0.0013	-
Average landings (kg per dredge track)	3.28	-	3.35	-
Average areal impact (m^2 per kg)	50	-	969	-
Løgstør Bredning	**2017**	**2018**	**2019**	**2020**
Average density of PO (kg m^2)	-	0.008	0.008	0.003
Average landings (kg per dredge track)	-	22.1	18.9	5.27
Average areal impact (m^2 per kg)	-	124	124	309

Lesson learned – feedback from stakeholder interviews

A series of telephone interviews with present and former fishers (a total of five fishers) with either licences for dredge fishery or hand collection of Pacific oysters in the Limfjorden and in the Wadden Sea was carried out. Furthermore, four traders were also interviewed of which three of them were also fishers. The interviews were structured by asking the same nine question and ten questions to fishers and oyster traders, respectively (the questions (in Danish) can be found in appendix 7.1).
In table 7.1.4, the feedback from each interviewed person is summarised. The overall experiences from the fishers were that the population of Pacific oysters especially in the deeper areas (>3 m) generally consists of too large individuals or clumps of Pacific oysters, to make a targeted fishery profitable in the Limfjorden and the Wadden Sea. Landings of Pacific oysters from the deeper areas are currently therefore caught as bycatch in the flat oyster and blue mussel fisheries in the Limfjorden. Hand collection of Pacific oysters in the Limfjorden and the Wadden Sea is generally evaluated as profitable and successful by both the fishers and the traders. However, there is a general concern that an increase in licences will result in an overexploitation of the right sized oysters, which would end the hand collection for years. A stable supply of hand collected Pacific oysters is important to be able to maintain the exiting market.

Table 7.1.4. Feedback summary of the stimulating and adverse aspects of previous experiences with Pacific oyster (PO) fisheries in Limfjorden or the Wadden Sea based on telephone interviews of present and former fishers and traders of the PO.

Fishers formerly with licence	Stimulating aspects	Adverse aspects	Future perspective
Fisher 1 – *had a licence for targeted dredge fishery*	By-catch in mussel/flat oyster dredge fishery, removing an invasive oyster without extra CO_2 emissions or environmental/bottom impact.	A targeted dredge fishery was not economic feasible e.g., large individuals or clumps – no possibility for selecting right sizes. Sorting of the catches was time consuming and detaching PO from clumps increased mortality.	Currently also a licence for hand collection. Sufficient supply of suitable/right sized PO for the fresh market – potential limitation in the future. Increase in licences might eliminate right sized PO in a few years. Currently, no future in targeted PO dredge fishery in the deeper areas - too big/clumps.
Fisher 2 – *only as bycatch*	PO caught as bycatch in mussel/flat oyster dredge fishery – removal of invasive species and getting minor payment.	Has not had a license but have considered it but in the deeper areas (>3 m) PO are too big or clumps.	Utilisation of non-commercial sizes would improve the feasibility and potentially make it more interesting/profitable.
Fishers currently with licence	**Stimulating aspects**	**Adverse aspects**	**Future perspective**
Fisher 3 - *Had licence for targeted fishery. Currently bycatch and hand collection*	Selling of bycatch is an extra income instead of a waste problem.	PO are large and clumps in the deeper areas and there is no market for them – fresh or processed that is economically viable.	Currently licence for hand collection. New tools to collect the PO in shallow areas could be of interest. Utilisation of large/clumps of PO are needed to initiating fishing/removal of large/clumps of PO in deeper areas.
Fisher 4 – *licence for hand collection*	Requests from traders are needed and consequently a stable supply to the traders for the fresh market is required.	Relative hard work to collect them by hand at <1 m. Only a few tons can be collected at a time. 300-600 kg collected/day Good quality as PO of the right sizes is selected.	Export of PO will require mechanical fishing in the shallow areas as upscaling would be needed.
Fisher 5 - *licence for hand collection and bycatch in flat oyster fishery*	Hand collection gives the opportunity to collect the right sizes which gives the highest prices.	Bycatches consist of large individuals or clumps. Are often waste and not an income. Only 30-50% of the catch can be sold.	Improved work conditions by mechanical fishing or tools in shallow – hand collection is too hard.
Fisher 6 – *licence for hand collection*	Target fishery of the right sized PO with a very good quality and taste.	Hard work which is not possible over longer periods.	Currently not actively collecting because it is too hard.

		Requires storage of PO in depots in the sea, which increased the risk of loss, decrease quality /meat content due to e.g., burial.	Pulling of smaller tools after a boat will be needed to improve the work environment
Traders	**Stimulating aspects**	**Adverse aspects**	**Future perspective**
Trader 1	Hand collection provide high quality PO, sold directly to restaurants or wholesalers for the domestic market as a local brand.	Storage in depots in the sea can have a negative effect on the quality. Preferable with land-based depots.	More PO can be sold but upscaling will require mechanical fishery e.g., mini-tools from a smaller dinghy with an outboard motor.
Trader 2	Branding "the good story" of hand collection as a sustainable and environmentally friendly fishery mitigating an invasive species often in collaboration with restaurants. Catches only consists of high-quality PO and right sizes (< 150 g). Danish oysters to same price as French oysters.	Often too big and general fast growth result in a lot of PO >150 g (upper limit for fresh market) and clumps of PO.	Good recruitments ensure sufficient supply of PO in the future but would need to harvest before PO get too big/start clumps. No interest in dredge/tools to collect PO, only hand collection due to the "good story". Potential risk of contamination in the shallow areas caused by overflow from sewers.
Trader 3	Smaller PO easy to sell when distribution is established (takes time). Developing the good story in collaboration with the restaurants – but with limitations since restaurants want a unique story to tell not mainstream. Sold to export.	Larger PO and clumps cannot be sold especially in the bycatches. Unstable supply chain of high-quality PO – improved by hand collection. PO are a natural resource so fluctuation in population size might jeopardise long term stable supply/risk of PO	Need for processing with increasing landings of PO (incl. the large/clumps of PO) as there is already a surplus for the fresh market.
Trader 4 (former)	Easy to sell hand collected PO for the fresh market (restaurants or shops). PO was high-quality (size and meat content).	Need for depuration in depots, which was not allowed by the authorities. Very little support/flexibility by the authorities.	No intention to start again until the regulations have been changed and collected PO can be kept in depots at sea until they are shipped.

The hand collection of Pacific oysters is generally hard work. It takes several days to collect a few tons and the right weather conditions are required. The working conditions were considered as the biggest disadvantage by the fishers but also as an obstacle for further upscaling of the hand collection. Increasing the landings of Pacific oysters will require development of e.g. new methods/tools that can be operated from smaller boats. However, this conflicts with the interests of the traders telling the

story of sustainable and low environmental impact fishery of invasive Danish Pacific oysters. Upscaling is therefore not seen as beneficial from the traders' perspective if i) hand collected Danish Pacific oysters are served in all restaurants (not a unique story), then the restaurants will not be willing to pay extra or ii) the sustainability and low environmental impact are compromised by initiating collection with tools from boats – as one of the traders stated, "dredge oysters are of no interest as the story changes completely". The relatively small quantities (few hundred kilos) that can be collected per day require that the Pacific oysters can be stored in depots until they are sold. Currently, both land-based and sea-based deports are in use. However, different challenges with sea-based depots have been expressed by the fishers/traders. Transfer of caught Pacific oysters in one shellfish production area[10] to a sea-based depot in another shellfish production area is not allowed, which currently prevent collection of Pacific oysters in some areas of the Limfjorden. Furthermore, in the Wadden Sea, the collected Pacific oysters must be stored in sea-based depots and can first be transferred to land-based depots after the opening sampling (cf. the EU food safety requirements) has been done. According to one of the traders, sea-based depots should not be allowed because it induces the risk of further spread of an invasive species.

7.2 Conclusion and Perspectives

All fisheries based on utilisation of wild resources are sensitive to fluctuations in the population but for Pacific oysters the fast growth and the gregarious behaviour is another often more challenging factor affecting the development of the population into less profitable from a commercial perspective. Initiatives to remove Pacific oyster by commercial utilisation have been of interest to the fishers in both the Limfjorden and the Wadden Sea for more than 20 years. However, it is first within the last five years a profitable fishery has evolved due to hand collection of Pacific oysters and development of marketing opportunities, benefitting also the bycaught Pacific oysters. Hand collection of Pacific oysters can be characterised as a "niche fishery" with a good story, as the Pacific oysters are mainly sold at a relative high price for the domestic fresh market but is challenged by the hard physical work environment. Based on the stakeholder feedback interviews, it does not seem like there is an interest from the current main traders in the Limfjorden to upscale the fisheries e.g. including hand-held tools operated from smaller boats, whereas in the Wadden Sea there is a strong wish from the fishers to improve the work environment by using a newly developed "oyster-collector tool" attached to a smaller boat instead of holding it by hand as currently allowed.

The factors identified as the main challenges for the further development of the Pacific oyster fishery in Denmark are:

- Hand collection of Pacific oysters of relatively small quantities of oysters requires the need for storage depots, either sea-based or land-based. Especially the sea-based depots have caused different problems in the Limfjorden and the Wadden Sea but the general perception of the fishers is that there is an inertia at the authorities to engage in a constructive dialog to solve the problems.

- Hand collection is hard work and time consuming, and an upscaling of the fishery will therefore require mechanical fishery and most likely new tools needs to be developed. However, implementation of mechanical fishery will require development of new markets – mainly export.

[10] Before molluscs can be sold the food safety requirements of Regulation (EC) 178/2002, Article 14 and more specific standards in Annex II of Regulation (EC) 853/2004 and the microbiological criteria adopted under Commission Regulation (EC) 2073/2005 must be met.

- Non-selective fishery tools increase the need for utilisation of larger individuals and clumps, which is already seen in the bycatch of Pacific oysters in other bivalve fisheries, where >50% of the catches often are considered as waste. To be able to utilise the “waste fraction” new processing methods, product development and export market need to be developed to improve both the profitability and the sustainability of the fishery.

- Development of a targeted fishery of Pacific oysters in the deeper areas (>3 m) in the Limfjorden is currently not of interest for the fishers because i) the population are dominated by larger individuals and clumps of little commercial interest and ii) the Pacific oysters in the deeper areas are mainly located within Natura 2000 sites, where the bivalve fisheries are regulated by an upper limit of 15% cumulative areal impact. The fishers therefore prioritise applying the “areal impact” to the profitable blue mussel and flat oyster fisheries in the Limfjorden. However, with the sharp reduction in the population of flat oysters in recent years, the Pacific oyster may be of greater interest to fishermen in the future.

The challenge with Pacific oysters of non-commercial interest in the deeper areas in the Limfjorden but also in the Wadden Sea could potentially be mitigated by initiating fisheries within delimited “fishing boxes” where areas are cleaned for Pacific oysters and where other bivalve species could be relayed to stimulate a development of individual Pacific oysters of the right commercial size in the areas. Thus, the fishery will help mitigate the effects of the invasive Pacific oyster in the areas.

7.3 References

Nielsen P, Nielsen MM, McLaverty C, Kristensen K, Geitner K, Olsen J, Saurel C, & Petersen JK (2021). Management of bivalve fisheries in marine protected areas. Marine Policy, 124, [104357]. https://doi.org/10.1016/j.marpol.2020.104357

8. Development, testing and environmental assessment of different new mitigation tools

The Pacific oysters have been known in Denmark since the 1970s due to introduction for aquaculture purposes multiple times e.g., the Limfjorden (1972), Isefjord (1986) and the Wadden Sea (1980s). The aquaculture production of Pacific oysters in Denmark stopped by the end-1990s (Nehls and Büttger 2007) but cultivation trials have occurred later[11]. Despite the ending of Pacific oyster farming, the species remained present in those areas, where it formed wild populations (Groslier et al., 2014, Holm et al., 2015, Wrange et al., 2010). The spread and increase in the wild populations in the three different areas varied. In the Wadden Sea, observations of dense populations were observed already in the 2007 (Kristensen & Pihl 2007), whereas in the Limfjorden, the observed densities were typically <3 ind m^{-2} in 2007 (Christensen & Elmedal 2007) and in Isefjord, the average density was 0.03±0.03 ind m^{-2} (Wrange et al. 2010). The further invasion of the Pacific oysters in the three areas are still very different (See section 2 for Limfjorden and Isefjord and Nielsen et al. 2018 for Wadden Sea) and often very site specific due to large variance in the populations structure of the Pacific oysters within the areas (c.f. section 2). Consequently, site specific mitigation initiatives would be needed to be able to remove the Pacific oysters in the best way both from an environmental and a fishery perspective.

In Denmark, mitigation of marine invasive species by e.g., programs for removal of established populations, is currently not implemented and it is the authors impression that it is very unlikely that such a mitigation program would be initiated by the national environmental authorities. Consequently, mitigations initiatives are driven by private interests – mainly by the fishery in areas with increasing wild populations of Pacific within the last >5 years (see section 7), which monetise the removal actions. However, the fisheries are challenged by multiple factors i) catches of oysters of non-commercial interest often becoming a waste problem, as discard of invasive species are not allowed, ii) Pacific oysters are mainly distributed in the very shallow (<1.5 m) areas, iii) clumps and reef structure are established within a few years (see section 2) and iv) Pacific oysters often co-exist with other bivalves, which increased the risk of bycatches. Where and when Pacific oyster populations significantly impact the shoreline and its communities, and conflict with other uses, is difficult to foreseen and whether removal of such oyster populations may be necessary.

Within this project the approach has been that one tool “does not fit all” and therefore three different mitigation tools have been tested; i) a mini-dredge for fisheries in shallow areas of individuals/smaller clumps of Pacific oysters have been developed and tested, ii) testing of a floating excavator to remove larger clumps/early established reefs and iii) a sorting equipment has been developed and tested to be able to sort mixed catches of blue mussels and Pacific oysters. To initiate mitigation programs or fisheries using one of the three mitigation tools listed above will require assessment of fishing efficiency, by-catch, and impacts on coastal community, which is presented below.

[11] Aquamind (2016) https://www.aquamind.dk/en/content/aquamind-tests-growth-conditions-pacific-oysters-isefjord. Acessed 12/07/2022.

Mitigation tool 1: Fishing efficiency and impacts of a new mini-dredge tool for removal of very shallow (<1.5 m) Pacific oyster populations in the Limfjorden

Authors: Pedro S. Freitas, Pernille Nielsen, Ciaran McLaverty and Camille Saurel

8.1 Introduction

Pacific oyster populations have expanded significantly in recent years in shallow habitats in Danish coastal waters. In the microtidal conditions of the Limfjorden, Pacific oysters have often established dense and reef populations at depths shallower than 1 m (Section 2). A mini-dredge was developed and tested and analysed with the following three aims:

1. Determine the fishing efficiency of the new mini-dredge tool when fishing for Pacific oyster in shallow areas, as well as the by-catch of non-target species, particularly of other bivalves. The fishing efficiency of both live and dead Pacific oyster, as well as isolated or clumped oysters was evaluated.

2. Assess the mini-dredge impacts on benthic macrofauna, macroflora and infauna from Pacific oyster fishing activities in shallow habitats (<1.5 m).

3. Assessment of the impact of the mini-dredge on eelgrass when the two species are co-ocurring.

Approach

A new mini-dredge tool for fishing of shallow Pacific oyster populations was developed by DTU Aqua (Figure 8.1.1), light and small enough to be operated by a single fisher on a small boat in shallow wates. After a testing period, three field trials were done: one to assess impact on an eelgrass bed and two to assess fishing efficiency and impact.

Figure 8.1.1. The new Pacific oyster mini-dredge tool (Photo: DTU Aqua).

8.2 Methods

Mini-dredge

The new mini-dredge tool was designed to scrape the bottom surface without digging into the bottom, having metal spikes at the bottom of the mouth that are maintained above the bottom surface by side skids, avoiding digging while pulling oysters sitting higher than the bottom (Figure 8.1.1). A spoiler was added to the top to help the dredge remain in contact with the bottom as the low weight of the dredge made it easily jump during testing. The bottom of dredge bag is made of metal rings and the back of bag is made of a rigid metal plate. The dredge mouth is 50 cm by x 18 cm and the bag is 50 cm long, for a total weight of 6.5 kg.

Field trials

After a period of testing and development, three field trials to simulate fishing activities were performed with the new Pacific oyster mini-dredge. A trial at a dense shallow Pacific oyster populations in Branden (Fur Sund) was used to determine fishing efficiency and impact on the benthic communities. The impact of the mini-dredge fishing on eelgrass was assess in a separate small trial on an eelgrass bed in Lysen Bredning (Figure 8.2.1). Field trials were carried using either the small DTU Aqua boat "Fjordrejen" or the small fishing boat "T51-ESSO" with the mini-dredge pulled with 12-14m of rope at the speed of 1-2 knots.

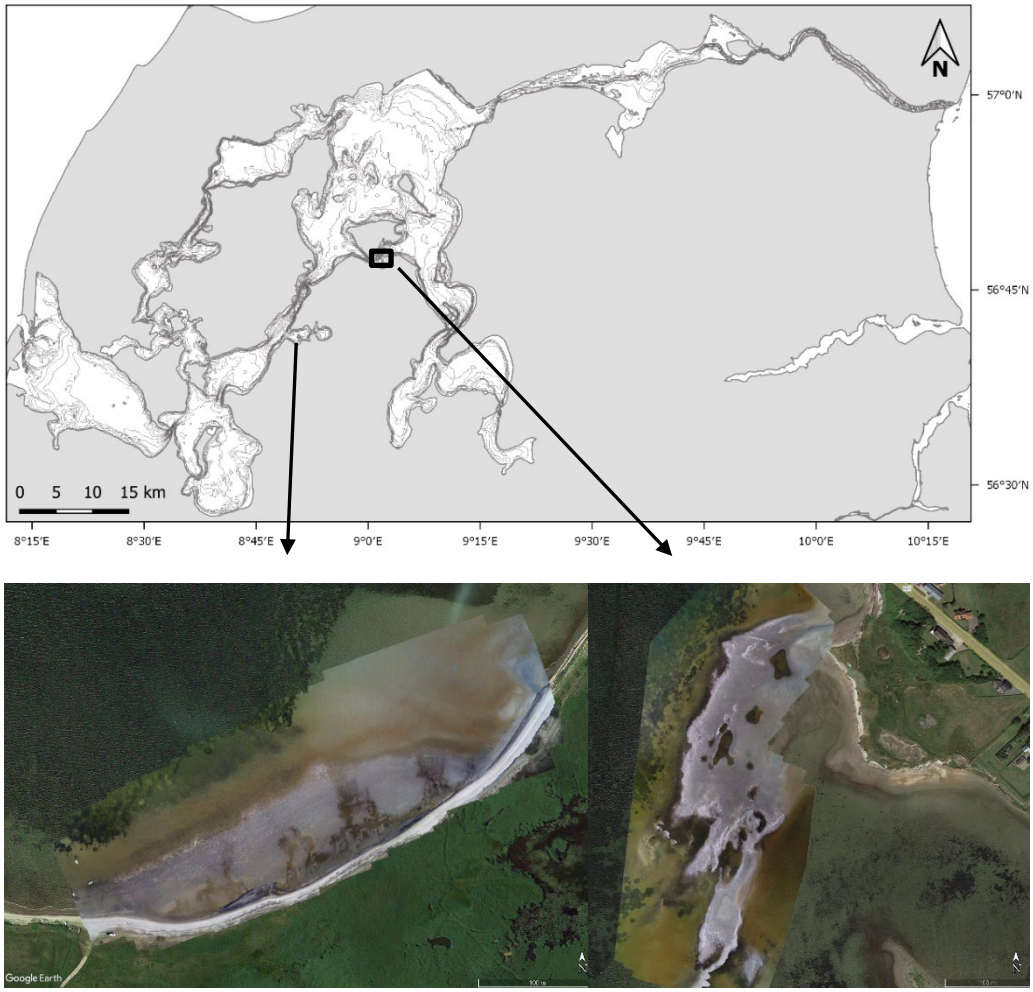

Figure 8.2.1. Fishing efficiency and trial impact areas in the Limfjorden: Lysen Bredning (southern rectangle) and Branden in Fur Sund (northern rectangle), Limfjorden (Google Earth).

Fishing efficiency trials

The fishing efficiency trial in Branden (Fur Sund, Figure 8.2.1 and 8.2.2) was done in November 2021, following a test and development period regarding the mini-dredge fishing behaviour in Lysen Bredning during the previous year. Fishing efficiency was assessed in two areas reflecting different population structure (Figure 8.2.2): A dense area (Dense) with high oyster density often with reef patches, constituted mainly of clumped oysters in a sand/mud bottom with some cobbles at ca. 40-50 cm depth; and an offshore lower density area (offshore) between the dense area and an eelgrass bed, made mainly of large isolated and clumped oysters over a sand/mud substrate at ca. 50-90 cm depth.

Figure 8.2.2. Fishing efficiency and impact trials in Branden Sund, Limfjorden were done in two areas with different Pacific oyster population structures: a high-density reef area (white structures on rectangles on the right) and lower density offshore area (left). In the impact trial dredge/impact areas (red rectangles) were compared to control areas (Yellow rectangles). Overlaid on a high-resolution drone image mosaic (Google Earth).

18 fishing tracks ranging between 16 and 22 m in length were dredged inside areas delimited with buoys in both the dense and offshore areas (Figure 8.2.2 and 8.2.3). After dredging, the dredge impacted areas were sampled with 50 by 50 cm quadrats (N = 15; Figure 8.2.2). All catch and quadrat samples were sorted for pacific oysters, macrofauna and macroalgae and measured in the laboratory. Fishing efficiency (F_i) was determined as % reduction in abundance (density or biomass) using mean catches per m^2 dredged (C) and mean abundance after dredging (A_d), with abundance before dredging equal to D_d +C: $F_i = C / (D_d + C)$.

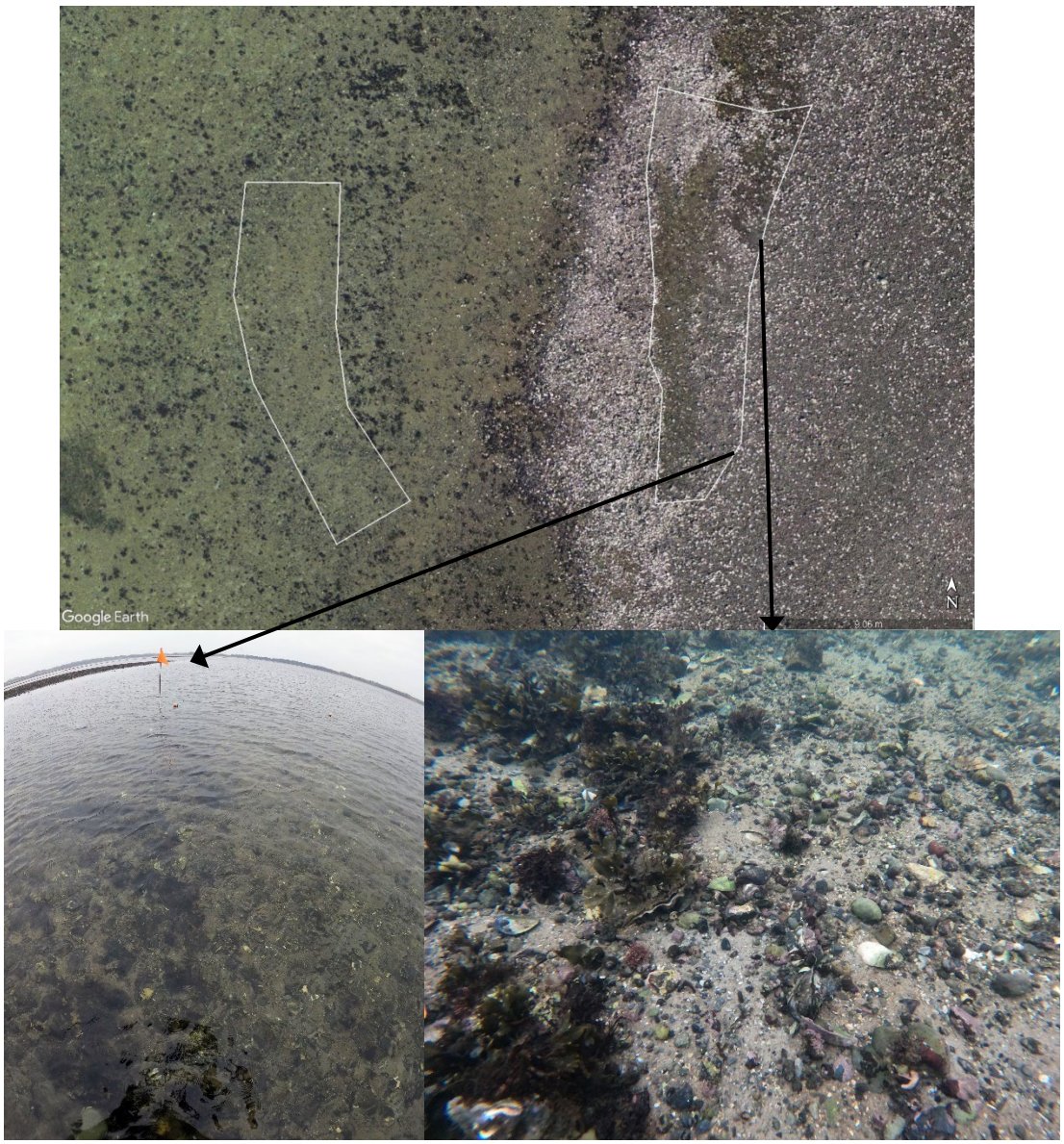

Figure 8.2.3. Post-dredging images of the dense population site. Top image: Drone mosaic (Aris Thomasberger, DTU Aqua) with dredge impacted sites in dense (right) and offshore (left) populations identified by white polygons, with clear visual differences relative to surrounding areas (overlaid in Google Earth). The dense population is partially emersed while offshore population is fully immersed, but nevertheless dredged tracks can clearly be identified in both populations: darker patches with no oysters in the dense population and bare, lighter patches in the offshore population. Bottom: Images of dense population during post-impact sampling. On left image, pole and buoys used to delimit dredged impacted area. On the foreground on the left and right images: Visual evidence of dredge impact with clear difference in macroalgae and Pacific oyster cover between a non-dredged area to the left and the dredge impacted area to the right (Google Earth, Photos Pedro Freitas, DTU Aqua).

Impact on macrofauna, macroflora and infauna

The impact from the mini-dredge on the macrofauna (excluding Pacific oyster), macroflora and infauna was assessed using a control-impact design on the same Pacific oyster populations as the fishing efficiency trial (Figure 8.2.2). Impact sites were the two dredged areas in the dense and offshore populations, with two control sites sampled in the immediate vicinity (Figure 8.2.2). The trial design assessed maximum impact as by-catch was landed and brought to the laboratory, while in fishing conditions by-catch would be discarded back to the local habitat, and thus partially reduce fishing impact.

Large macrofauna and macroalgae were sampled using quadrats (0.5 by 0.5 m, 0.25 m^2) as described for fishing efficiency (N = 9 in each site). Macrofauna was described as abundance (number or weight of individuals) of major taxa, while for macroalgae only the total weight of all species was measured.

Samples of smaller macro-epifauna and infauna were collected using a HAPS core (KC Denmark; area 0.0145 m^2, N = 9 in each site) after removal of Pacific oysters and associated macroalgae. Samples were sieved with a 1mm sieve, preserved in 70% ethanol, and later sorted and analysed under a binocular microscope in the laboratory.

Figure 8.2.4. Trial area (white polygon) in Lysen Bredning for evaluating fishing impact of the new mini-dredge on eelgrass. Five tracks of 30-35m length were dredged on a large eelgrass bed next to a large Pacific oyster population (bottom of image). High-resolution drone image mosaic (Pedro Freitas, DTU Aqua) overlaid on Google Earth.

Community composition was described as abundance (number or weight of individuals), species richness (number of species), Shannon's diversity (H) and evenness (E_H) indices: $H = -\Sigma(p_i * \ln p_i)$, where Σ is the sum of the natural log of pi, the proportion of the entire community made up of species i; $E_H = H / \ln(S)$ where, H is the Shannon Diversity Index and S is the total number of unique species. E_H ranges from 0 to 1 and 1 indicates complete evenness. It must be noted that highly mobile fauna, e.g. fish, shrimps and crabs, can move away and likely avoided sampling.

Mini-dredge impact was assessed as changes in species richness, abundance and community composition between control and impact sites using multivariate analysis. Data were log transformed, and resemblance matrices calculated using Bray-Curtis similarities. Cluster analysis, non-metric multidimensional scaling (NMDS) and multivariate Anova (Permanova) were carried out to test if community composition (or species composition) differed between control and impact sites separately for dense and offshore populations. SIMPER (similarity percentage) analysis was done to identify which species made the greatest contribution to observed dissimilarity between groups. Regarding the main macrofauna species and macroalgae sampled with quadrats, conclusions from multivariate analysis of community composition require caution due to the low number of species (0 to 4) or individuals (0 to 91) present in any given replicate (Table 1 appendix 8.1).

Impact on eelgrass

A fishing impact trial was done in a large eelgrass bed in Lysen Bredning adjacent to a large and dense Pacific oyster bed (Figure 8.2.1 and 8.2.4). Five 30-35 m long tracks were dredged with the mini-dredge tool filled with Pacific oysters to 25% capacity to simulate realistic fishing conditions.

Eelgrass fishing efficiency/impact was estimated by two methods: 1) a visual diver assessment of fishing impact (F_v) as % reduction in eelgrass cover from pairwise adjacent quadrats inside (E_i) and outside (E_o) dredge tracks (Figure 8.2.5): $F_v = (E_o - E_i) / E_o$. This approach reflects changes in eelgrass biomass and eelgrass leaves. 2) A catch-abundance approach, where fishing impact (F_a) was estimated from mean eelgrass dredge catch (C) and mean eelgrass density in non-dredged areas (D): F = C / D. Eelgrass density was determined by quadrat sampling (50 x 50 cm, N = 12) outside dredge tracks from the number of eelgrass roots, which are unaffected by seasonal changes in biomass due to growth or shedding of leaves.

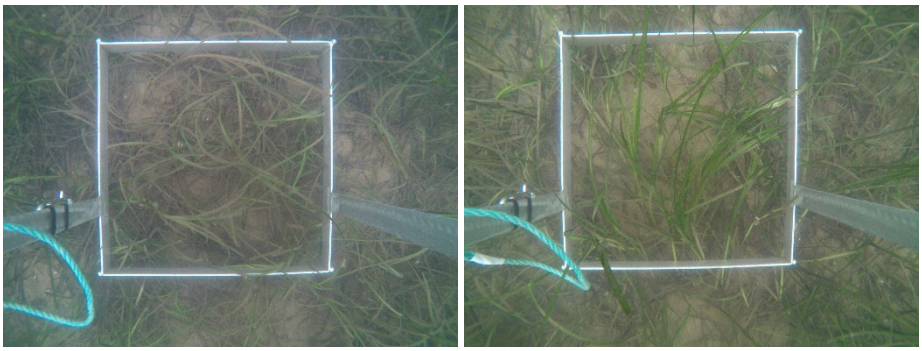

Figure 8.2.5. Examples of quadrat sampling of eelgrass cover and root density outside (left) and inside (right) of the dredge tracks. Notice the clean aspect of the eelgrass leaves inside the dredge tracks due to the action of the dredge and its bag on eelgrass leaves (Photos: Daniel Taylor, DTU Aqua).

8.3 Results

Fishing efficiency

Mini-dredge catches

For live Pacific oysters (Figure 8.3.1, Table 1, appendix 8.1), the number of live oysters caught was significantly lower in the dense area at 0.86 ±0.1 (SE) oysters/m^2 than in the offshore area at 2.08 ±0.4 (SE) oysters/m^2 (Kruskal-Wallis non-parametric test, $H = 13.24$, $p = 0.0003$). The catch of individuals was lower in the dense than offshore area (KW, $H = 10.41$, $p = 0.0013$), while catches of clumped oysters where not different between the two areas (KW, $H = 1.67$, $p = 0.167$). Regarding dead oyster shells (Figure 8.3.1; Table 2 appendix 8.1), catches were significantly higher in the dense area than offshore area, 10.8 ±1.3 relative to 0.2 ±0.1 (SE) oysters/m^2 (KW, $H = 22.87$, $p < 0.0001$).

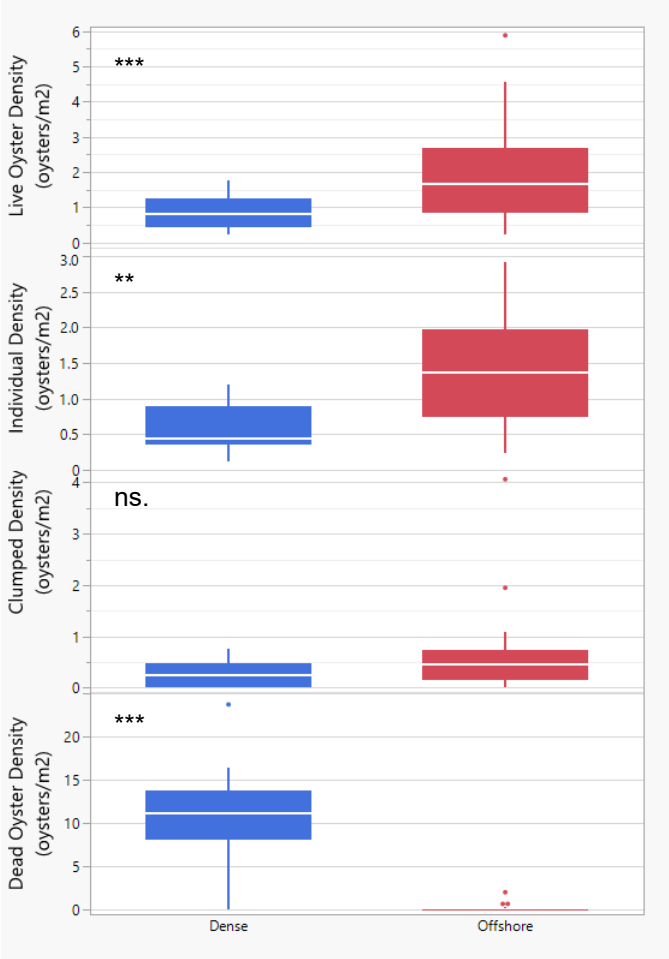

Figure 8.3.1. Total live pacific oyster catches, the divided into individual and clumped oysters, and dead Pacific oyster catch (left, oysters/m^2 dredged) in the dense (blue) and offshore areas (red). Asterisk indicates level of significance in differences: * <0.001, ** <0.01, * <0.05, ns. – not significant.**

Live Pacific oyster catches by weight were significantly lower in the dense than offshore area (KW, *H* = 12,63, *p* = 0.0004) at 119 ±22 (SE) g/m^2 in the dense area and 479 ±94 (SE) g/m^2 in the offshore area (Figure 8.3.2, Table 1 appendix 8.1).

Total dredge catches were significantly higher in the dense than offshore area (KW, *H* = 12.33, *p* = 0.0004) at 1 579 ±140 (SE) g/m^2 in the dense area and 822 ±109 (SE) g/m^2 in the offshore area (Figure 8.3.2, Table 1 appendix 8.1).

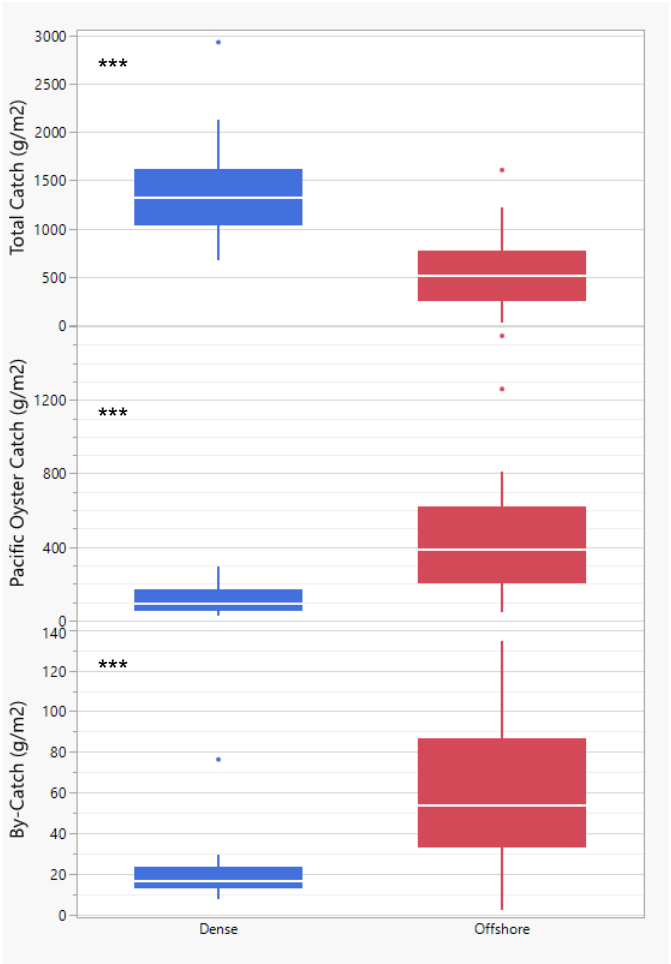

Figure 8.3.2. Total biomass catch, Pacific oyster biomass catch and by-catch biomass of live organisms (left, g/m^2 dredged) by the dredge in the dense (blue) and offshore areas (red). Asterisk indicates level of significance in differences: * <0.001, ** <0.01, * <0.05).**

By-catch

By-catch of live organisms was significantly lower in the dense than offshore area (KW, H = 15.89, p < 0.0001), at 20 ±4 (SE) g/m^2 in the dense area and 61 ±9 (SE) g/m^2 in the offshore area (Figure 8.3.2, Table 1 appendix 8.1). The dead shells dominated the by-catch by weight (Figure 8.3.3, Table 1 appendix 8.1), being significantly higher in the dense than in the offshore area (KW, H = 16.70, p < 0.0001). Regarding by-catch of live organisms (Figure 8.3.3, Table AP1), by-catch of bivalves and macroalgae was lower in the dense than in the offshore area (KW, H = 17.44, p < 0.0001 and H = 8.29, p = 0.0040), while the reverse occurred for other invertebrates that were lower in the offshore area (KW, H = 14.97, p < 0.0001).

Bivalve by-catch in both areas was entirely made of the blue mussel *Mytilus edulis,* with a single carpet clam (*Venerupis pullastra*) caught in one dredge. Other macroinvertebrates in the dense area were mainly the common periwinkle (*Littorina littorea*, 0-2.7 individuals/m^2 dredged), with minor occurrence of ascidians (*Ciona intestinalis*, *Ascidiella sp.*) and polychates (non-identified), all absent from the offshore area. Other macroinvertebrate species, such as the shore crab (*Carcinus maenas*), common starfish (*Asterias rubens*), sea urchin (*Psammechinus miliaris*), ascidians (*Ciona intestinalis*, *Ascidiella sp.*) and polychates (non-identified) were normally absent in both areas (only in 0 to 12.5% of dredges) and never more than 5 individuals were caught in any dredge (0.1-0.7 individuals/m^2 dredged). A single vertebrate, a goby (*Gobiidae sp.*), was caught trapped in an oyster shell by one dredge in the dense area.

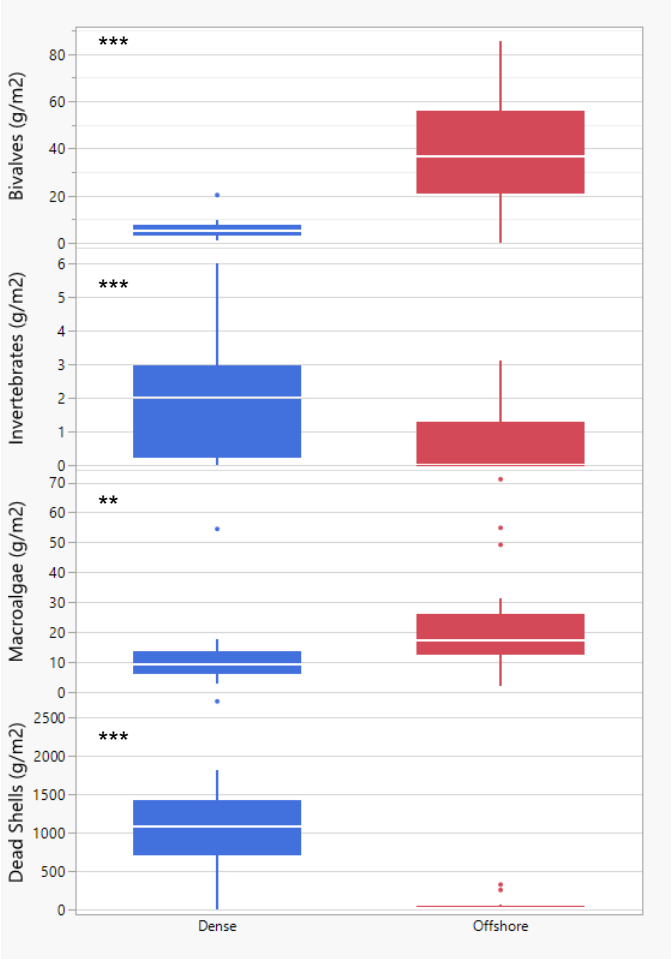

Figure 8.3.3. Bivalves, other invertebrates, macroalgae and dead shells in the by-catch by weight (g/m^2 dredged) in the dense (blue) and offshore areas (red). Asterisk indicates level of significance in differences: * <0.001, ** <0.01, * <0.05).**

Pacific oyster fishing efficiency

The fishing efficiency of the mini-dredge for live Pacific oysters, i.e. % reduction in abundance from dredging, was 49.1 ±33% (SE) in the dense area and 82.4 ±84% (SE) in the offshore area (Table 8.3.1). For individual Pacific oysters, fishing efficiency was 39.5 ±27% (SE) in the dense area and 76.0 ±77% (SE) in the offshore area, while for clumped oysters it was 100% in both areas (Table 8.3.1). The large variability (SE) implied no difference was observed in Pacific oyster fishing efficiency of the mini-dredge between the two areas.

Table 8.3.1. Pacific oyster fishing efficiency as % removed by the mini-dredge (± SE) in the dense and offshore areas.

	Fishing Efficiency (%) Pacific Oysters			
Area	Live	Dead	Individual	Clumps
Dense	49.1 (±33)	40.3 (±11)	39.5 (±27)	100
Offshore	82.4 (±84)	100	76.0 (±77)	100

Impact on macrofauna, macroalgae and infauna

The mini-dredge impact on the benthic community was assessed in two separate approaches: 1) for the six macro- epifauna species present, i.e. Pacific oysters *C. gigas*, large blue mussel *Mytilus edulis*, periwinkle *Littorina littorea*, slipper limpet *Crepidula fornicata* shore crab *Carcinus maenas,* starfish *Asterias rubens,* and for macroalgae combined as whole. These were sampled with quadrats; 2) for smaller macro- epifauna and infauna, excluding Pacific oysters and macroalgae. These were sampled with cores.

Major Macrofauna and macroalgae

Dense vs offshore populations: Control sites

Neither the abundance of live Pacific oysters (KW, H = 3.01, p = 0.083), macro- epifauna species richness (number of species) or Shannon's diversity index (KW, H = 2.16, p = 0.142 and H = 0.34, p = 0.562; Figures 8.3.4 and 8.3.5) were significantly different in the dense and offshore control sites. A maximum of 4 species were present in any given sample (Table 3 appendix 8.1) and only a total of 5 different species were identified. Abundance was never more than 91 individuals in any sample, but usually fewer than 40-50 individuals (Table 3 appendix 8.1).

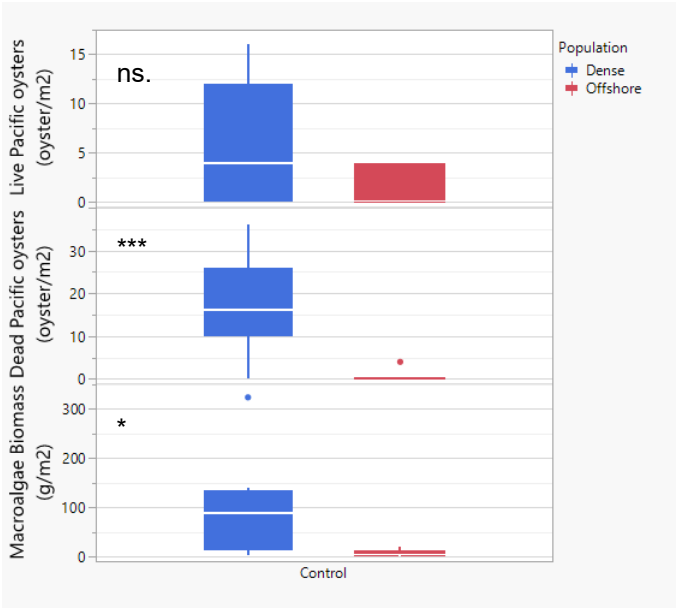

Figure 8.3.4. Abundance of live and dead Pacific oysters and macroalgae in control dense (blue) and offshore sites (red). Asterisk indicates level of significance in differences: * <0.001, ** <0.01, * <0.05, ns. – not significant.**

Only the abundance (number of individuals) and macroalgae biomass, usually attached to live or dead oyster shells, were higher in the dense than in the offshore control site (KW, H = 12.84, p = 0.0003 and H = 6.58, p = 0.0103, respectively; Figures 8.3.4 and 8.3.5). PERMANOVA test indicated significant differences between the dense and offshore control sites (p = 0.0002) in both the dense and offshore populations.

The presence of reefs in the dense population was reflected in a higher abundance of dead Pacific oyster shells (KW, H = 11.24, p = 0.0008) by two orders of magnitude than in the offshore site (17.8 ±3.7 oyster/m^2 and 0.4 ±0.4 oyster/m^2, respectively; Figure 8.3.4). Therefore, mini-dredge impact was assessed separately in the dense and offshore populations, even though abundance of live Pacific oysters was similar, as the structure of the Pacific oyster populations is different with the presence of reefs in the former (Figure 8.2.2).

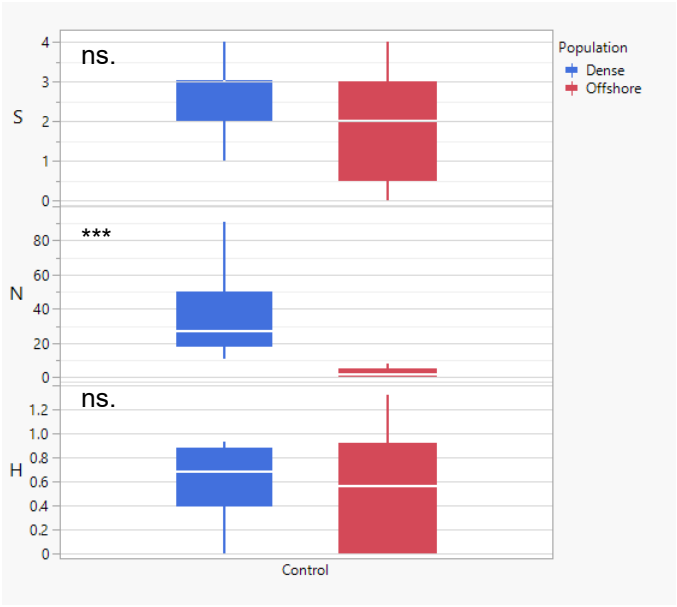

Figure 8.3.5. Species richness (S), abundance (N) and Shannon's diversity index (H) in control sites of dense (blue) and offshore populations (red). Asterisk indicates level of significance in differences: * <0.001, ** <0.01, * <0.05, ns. – not significant.**

Differences in individual species

A total of six major macro-epifauna species were identified, five in the dense population and six in the offshore population (Table 3 appendix 8.1). Abundance was higher at the dense (N = 481) than at the offshore population (N = 67). The only significant impacts observed were for macroalgae biomass and Pacific oyster abundance in the dense population, which were significantly higher in the control site than in the impact site (KW, H = 9.03, p = 0.0027 and H = 4.26, p = 0.0391, respectively; Figure 8.3.6).

No other differences were observed for macroalgae biomass and live Pacific oyster abundance in the offshore population or in blue mussel (*M. edulis*), shore crab (*C. maenas*), starfish (*A. rubens*), periwinkle (*L. litorea*) or slipper limpet (*C. fornicata*) abundances between control and impact sites in both the dense and offshore populations (KW, all H <2.18, p >0.05; Figure 8.3.6).

Differences in community

No significant difference was observed in species richness, abundance and diversity between control and impact sites in both the dense and offshore populations (KW, all H <3.62, p >0.05; Figure 8.3.7).

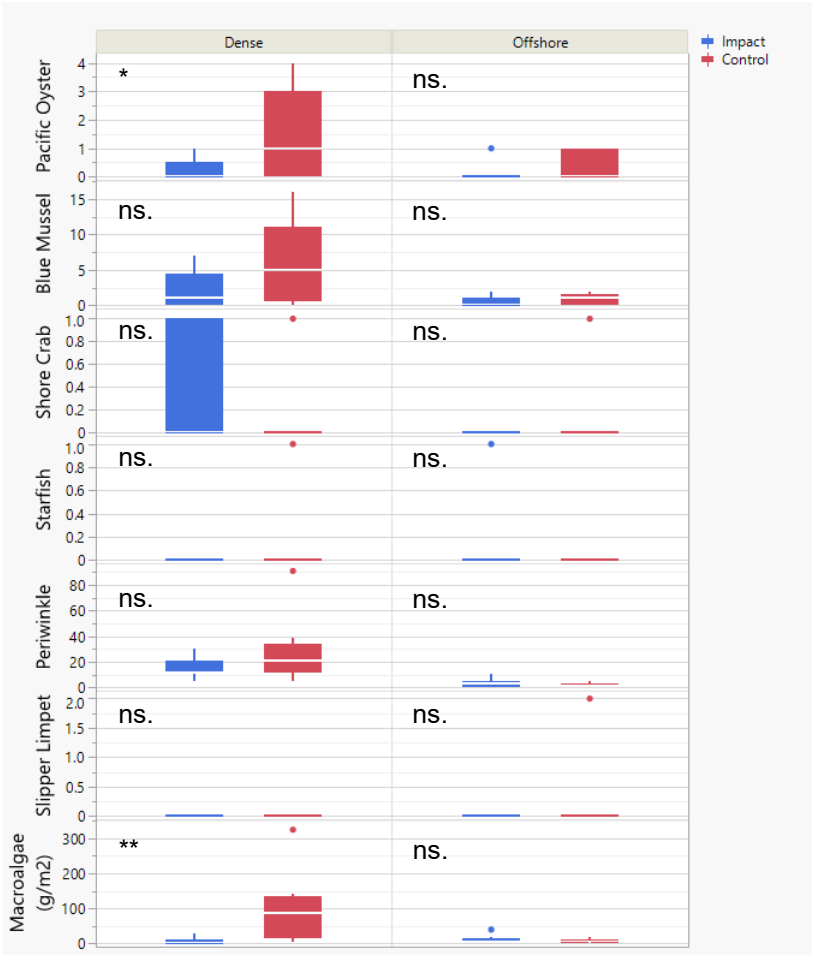

Figure 8.3.6. Large major macrofauna species and total macroalgae biomass in control (red) and impact (blue) sites of dense (left) and offshore (right) populations. Asterisk indicates level of significance in differences: * <0.001, ** <0.01, * <0.05, ns. – not significant.**

Clustering analysis showed that species composition did not differ between the impact and the control sites in both the dense and offshore populations (Figure 8.3.8). Clusters were not linked to control and impact sites, but to the presence of Pacific oysters and blue mussels in the dense population and periwinkle in the offshore population (Figure 8.3.8). This was confirmed in non-metric multidimensional ordination plots (nMDS), which showed no clear differences between control and impact sites in the dense and offshore populations, with no significant clusters and thus overlapping 95% confidence ellipses (Figure 8.3.9). PERMANOVA test indicated no significant differences between the control and impact sites ($p > 0.05$) in both the dense and offshore populations.

Figure 8.3.7. Species richness (S), abundance (N) and Shannon's diversity index (H) in control (red) and impact (blue) sites of dense (left) and offshore (right) populations. Asterisk indicates level of significance in differences: * <0.001, ** <0.01, * <0.05, ns. – not significant.**

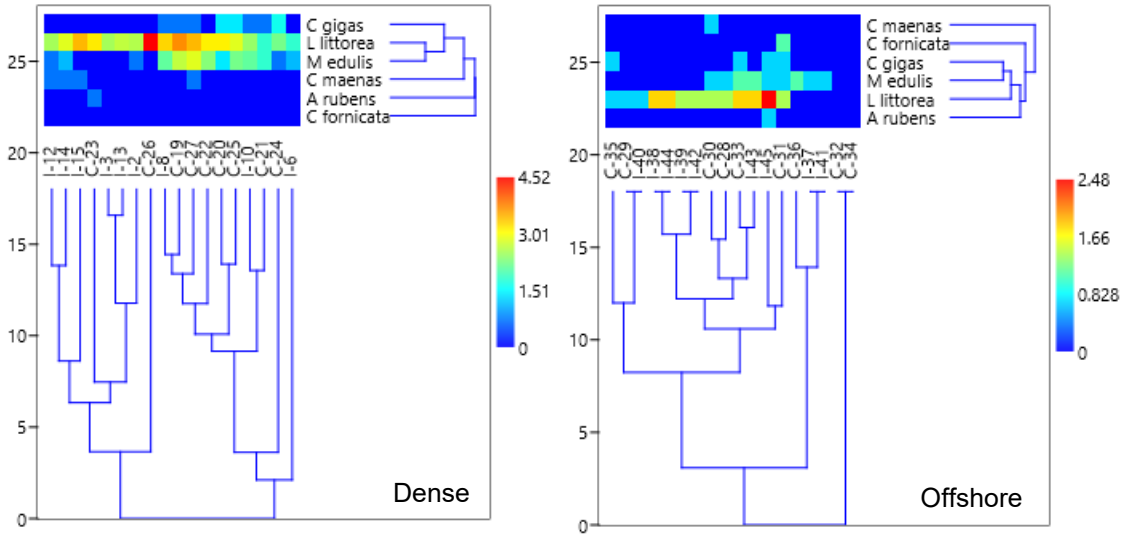

Figure 8.3.8. Clustering plots from Bray-Curtis similarities for control and impact sites in the dense (l eft) and offshore (right) populations. Top plot indicates relative abundance of species at each replicate with control and impact sites identified by C and I prefixes.

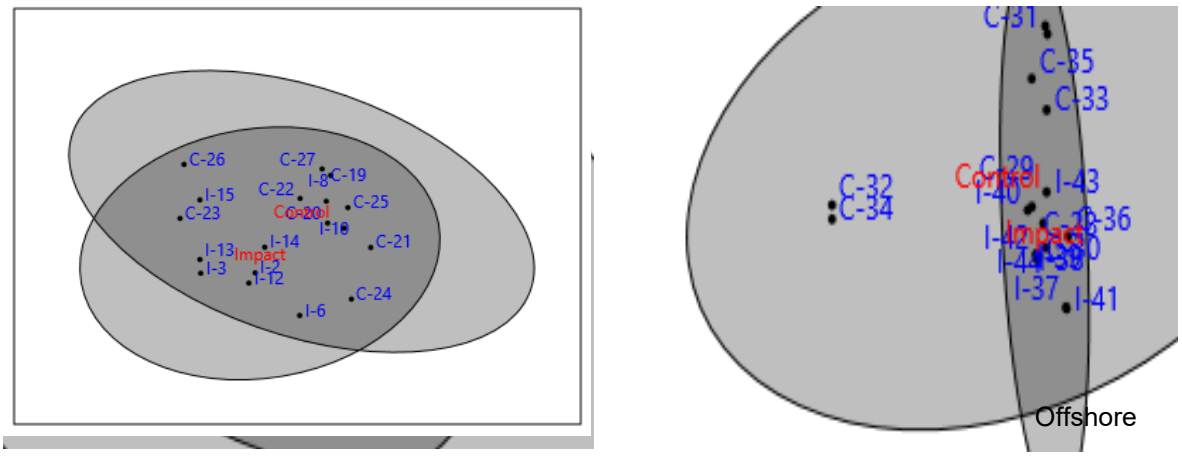

Figure 8.3.9. Non-metric multidimensional ordination plots (nMDS) from Bray-Curtis similarities for control and impact sites in the dense (left) and offshore (right) populations. Control and impact replicates identified by C and I prefixes. Ellipses identify significant clusters.

Small epifauna and infauna

Dense vs offshore populations: Control sites

A total of 55 taxa were identified, with 44 found in the reef site and 39 recorded at the offshore site. In terms of abundance, it was higher at the dense site (N = 3279) compared to the offshore site (N = 787).

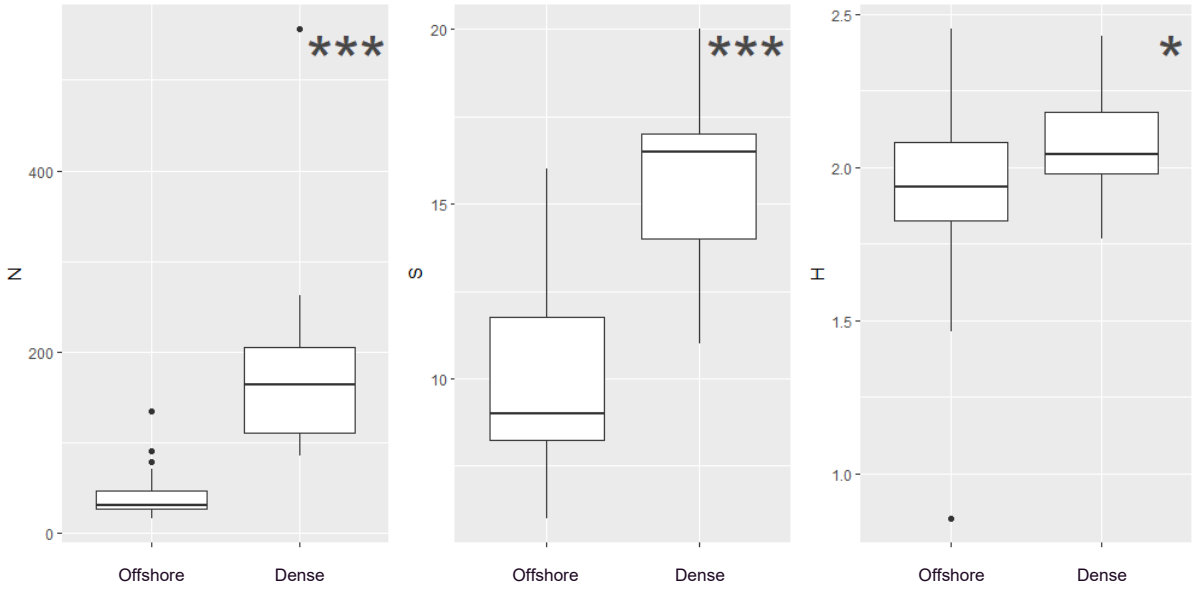

Figure 8.3.10. Box plots of small epifauna and infauna species richness (S), abundance (N) and Shannon's diversity index (H) in control sites of dense (blue) and offshore populations (red). Asterisk indicates level of significance in differences: * <0.001, ** <0.01, * <0.05.**

Examination of the dense and offshore populations were compared to determine if mini-dredge fishing impacts could be analysed together and the examination indicated that there were significant differences (Figure 8.3.10, table 4 appendix 8.1) between populations in in abundance (N), species richness (S), and Shannon diversity (H): N (p = <0.001), S (p = <0.001), and H (p = 0.047).

Clustering analysis showed species composition differed between control sites of the dense and offshore populations, with the dense populations showing two distinct clusters (Figure 8.3.11). Non-metric multidimensional ordination plots (nMDS) confirmed the difference between control sites of the two populations (Figure 8.3.12). Permanova analysis confirmed that community (or species composition) of control sites differed between the dense and offshore populations (p = 0.001). Therefore, as for the major macro-epifauna and macroalgae, the impact of the mini-dredge on dense and offshore populations was analysed separately.

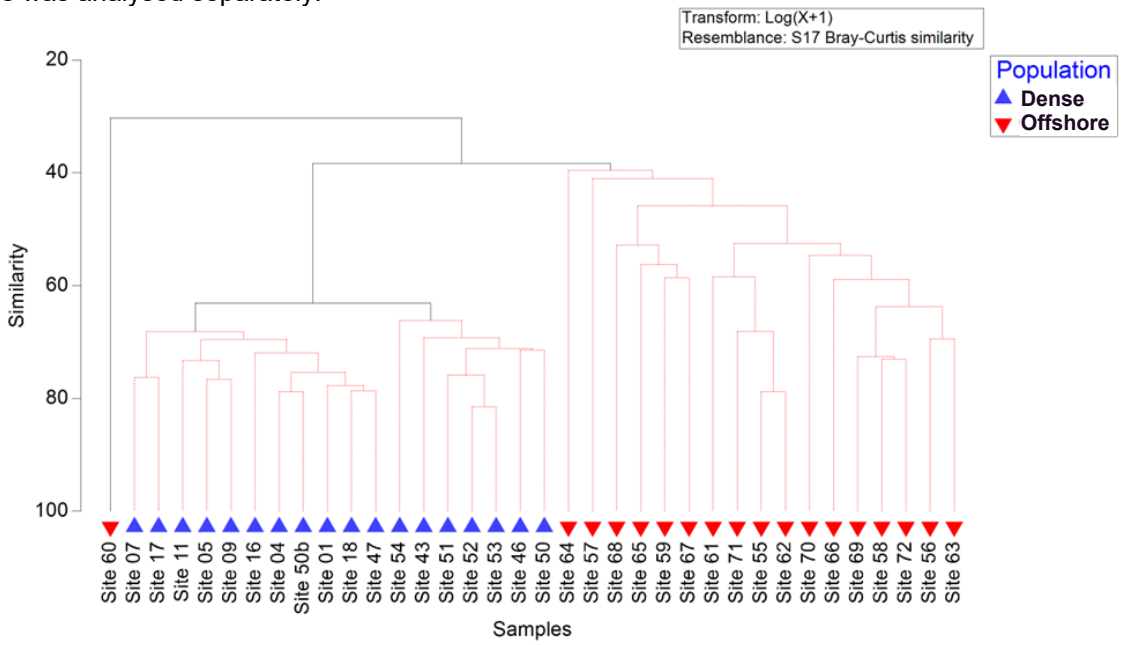

Figure 8.3.11. Clustering analysis from Bray-Curtis similarities between control sites of dense (blue) and offshore (red) populations. The red lines show the statistically significant site clusters, with the black line showing where they are different.

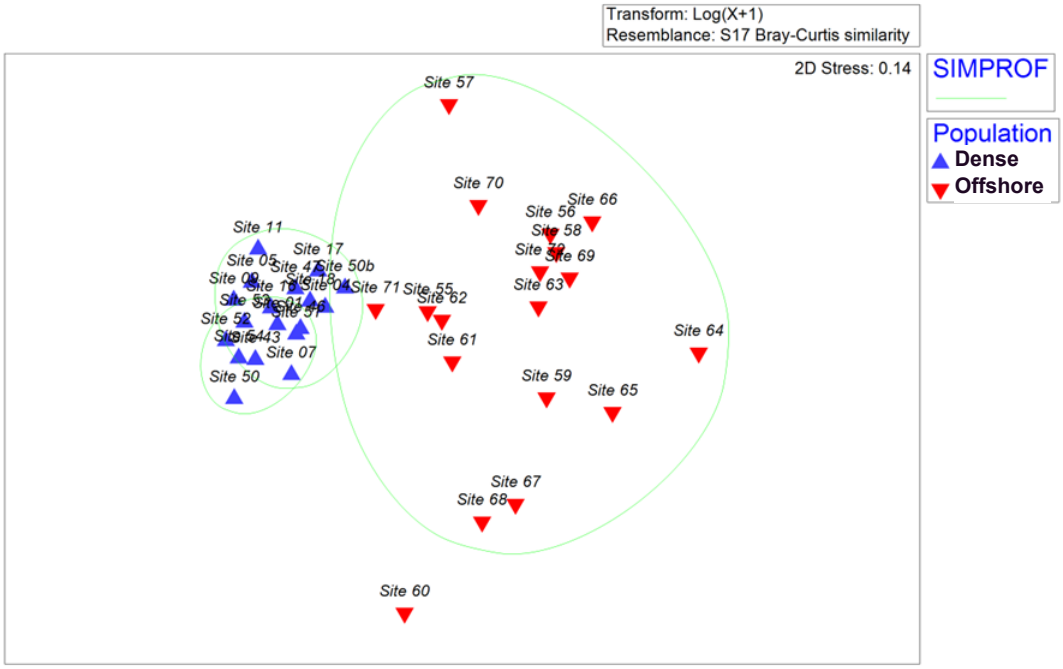

Figure 8.3.12. Non-metric multidimensional ordination plots (nMDS) from Bray-Curtis similarities for control sites in the dense (blue) and offshore (red) populations. The green line shows statistically significant site clusters.

Differences in community
Significantly less abundance and species richness were recorded in the impact treatment (Figure 8.3.13), however, Shannon diversity showed the opposite trend, and was higher in the impact treatment (although non-significant). There were no significant differences in abundance, species richness and Shannon diversity between the control and impact treatments in the offshore area (Figure 8.3.13). The trend was, however, like the reef with abundance and species richness lower and Shannon diversity higher in the impact site.

Clustering analysis showed communities in dense populations at the impact site were significantly different from the control site (except for 2 replicates), while at the offshore population communities were similar, with no differences between the control and impact sites (Figure 8.3.14). Non-metric multidimensional ordination plots (nMDS) confirmed the difference between control and impact sites at the dense population, but not at the offshore populations (Figure 8.3.15).

Permanova analysis confirmed that community (or species composition) of control sites differed from impact sites at the dense population ($p = 0.003$), but not at the offshore population ($p = 0.718$). SIMPER analysis of similarity evaluated which species contributed most to the observed dissimilarity between the control and impact sites at the dense population, only. Firstly, in terms of the similarly of sites within control and impact sites separately (Table 8.3.2), and then finally at the species responsible for the differences between control and impact treatments (Table 8.3.3). In the impact site, 5 species account for roughly 76% of the similarity. Of these, the bivalve *Mytilus edulis* and the oligochaete *tubifcoides* sp. were most common across replicates, and therefore were associated with the largest contributions (accounting for roughly 30% and 16% of the similarity between replicates).

In the control site, 3 species account for roughly 73% of the similarity of replicates (and confirms the lower evenness observed in Table 4 Appendix 8.1). Again, *Mytilus edulis* was an important species in terms of abundance and similarity (30%) across sites in the control treatment, but with the mud shrimp *Corophium* sp. also being of roughly equal importance (27%).

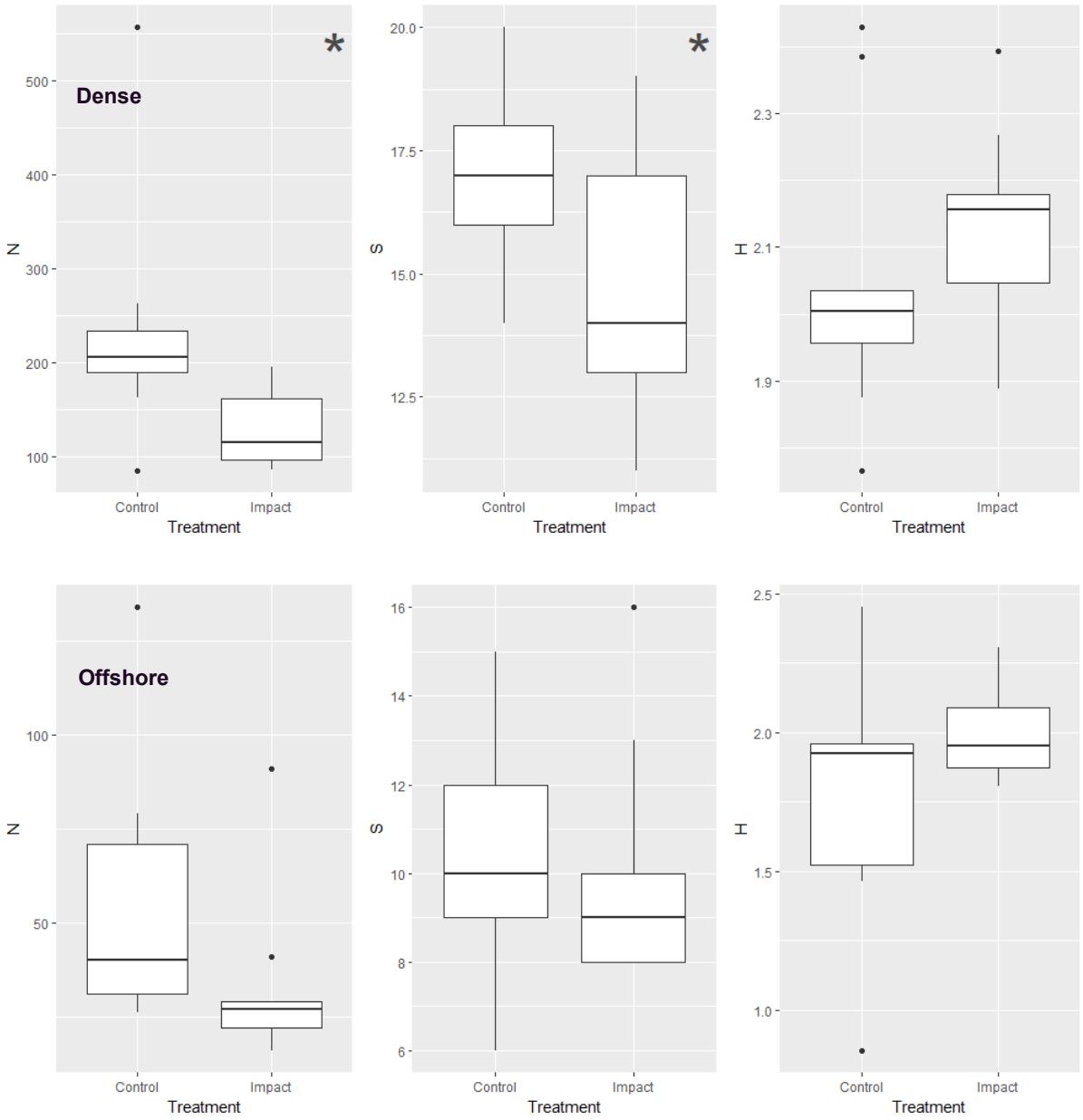

Figure 8.3.13. Box plots of small epifauna and infauna species richness (S), abundance (N) and Shannon's diversity index (H) in control and impact sites of dense (top) and offshore populations (bottom). Asterisk indicates level of significance in differences: * <0.001, ** <0.01, * <0.05.**

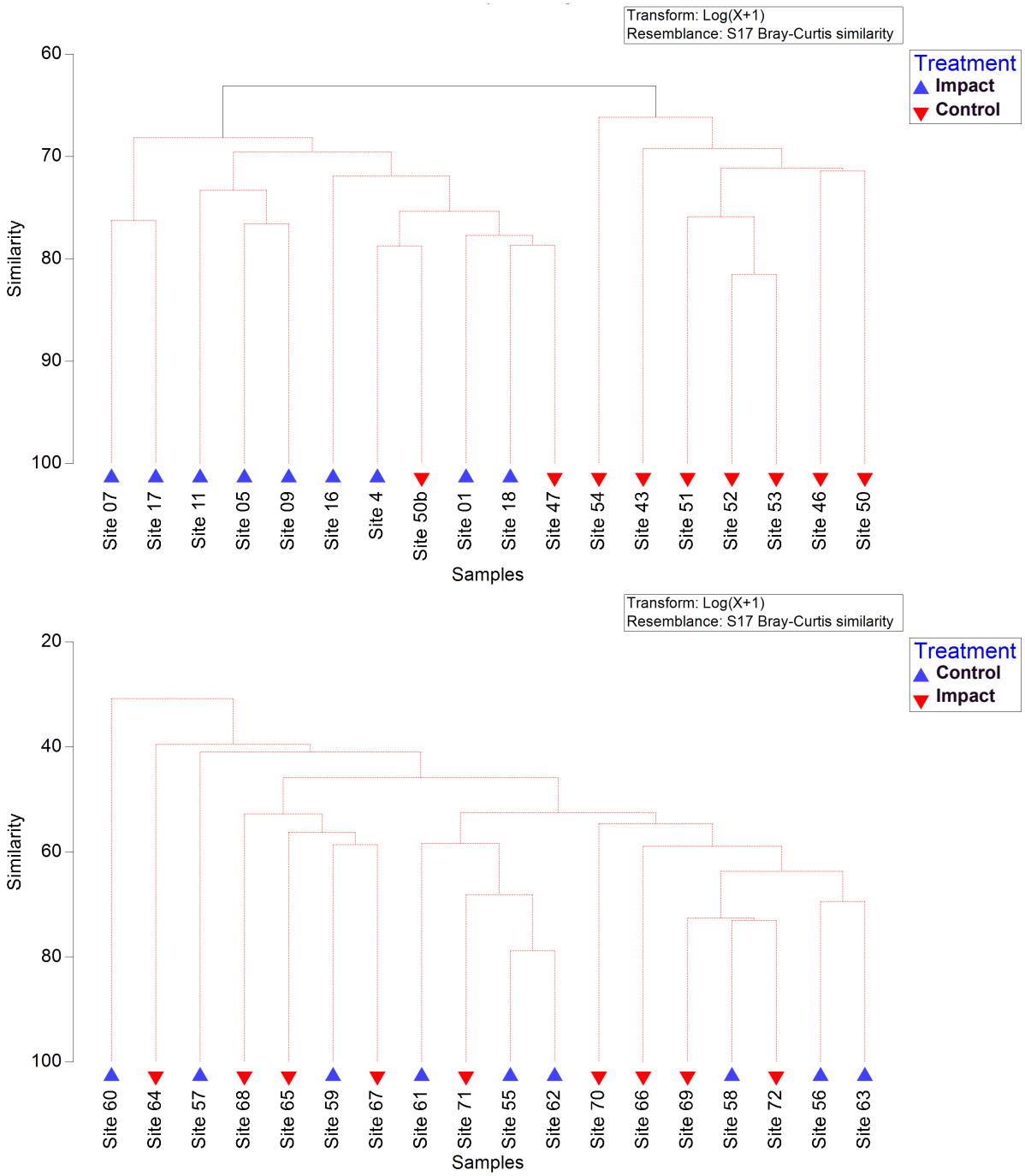

Figure 8.3.14. Clustering analysis from Bray-Curtis similarities between control and impact sites of dense (top) and offshore (bottom) populations. The red lines show the statistically significant site clusters, with the black line showing where they are different.

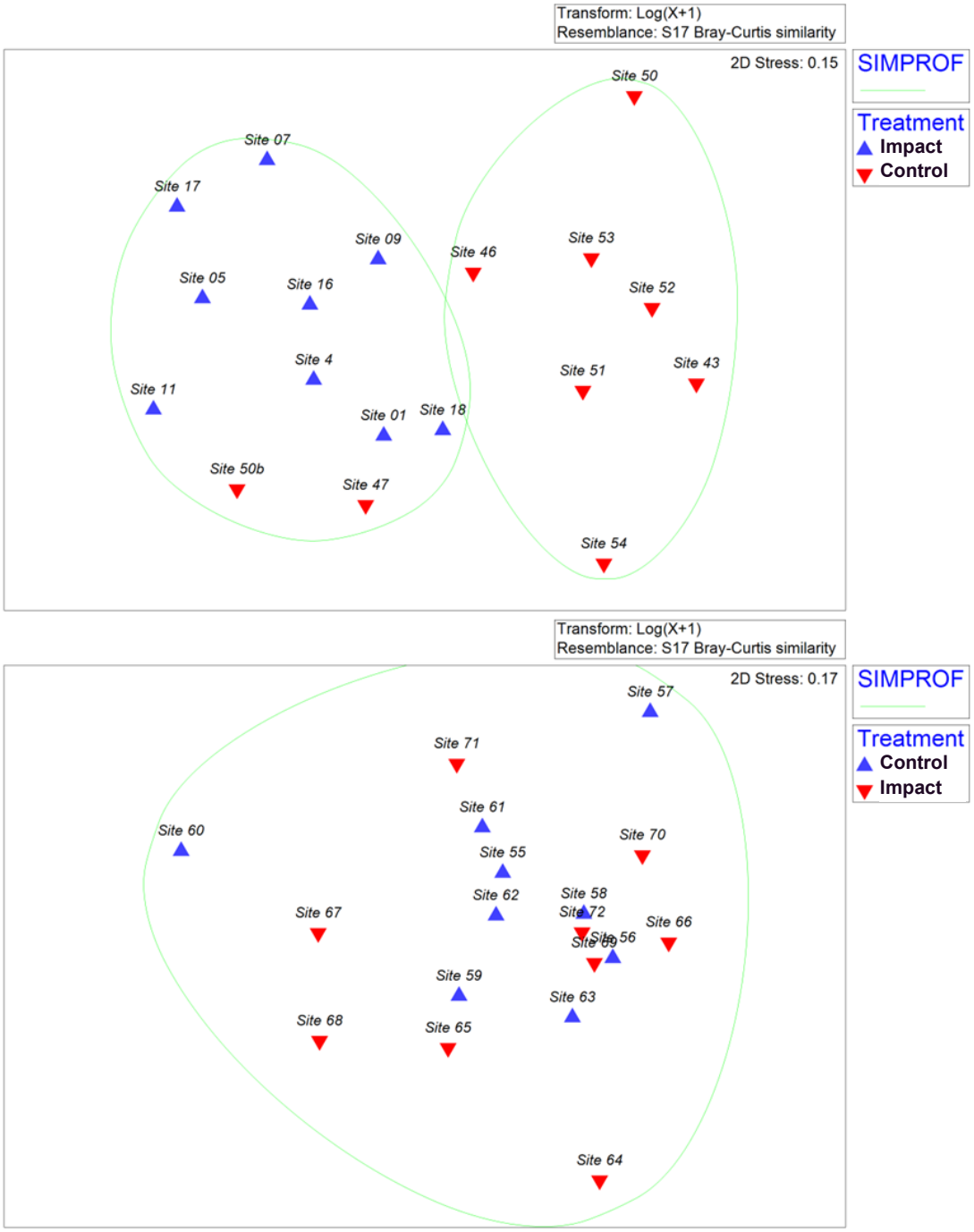

Figure 8.3.15. Non-metric multidimensional ordination plots (nMDS) from Bray-Curtis similarities for control sites in the dense (blue) and offshore (red) populations. The green line shows statistically significant site clusters.

In terms of the differences between control and impact sites, six species account for the majority (73%) of dissimilarity between the sites. Of these, the mud shrimp *Corophium* sp. made the greatest contribution (19%) to differences between sites. This was as the abundance of *Corophium* sp. was much higher in the control site, but also as the species was common to sites in both control and impact sites. This resulted in a high contribution and therefore high importance. *Mytilus edulis* was also an important species underpinning differences between sites. It was found in higher abundances in the control site, likely due to it being removed in the dredged area and consequently it associated with a high contribution (15%) towards differences between sites. Species such as *Balanus* sp., *Tubi-*

fcoides sp. and *Spiophanes* were also more abundant in the control site, with these 3 species also accounting for ~33% of the differences between control and impact sites. The amphipod *Gammarus salinus* differed as it was found in higher abundance in the impact site and accounted for a relatively small amount (5%) of differences between control and impacted sites.

Table 8.3.2. SIMPER analysis of contribution of individual species to the observed similarity within the impact and control sites separately.

Main species for impact site Average similarity: 59.35					
Species	Average Abundance	Average Similarity	Sim/SD	Contribution %	Cumulative %
Mytilus edulis	30.56	18.19	4.39	30.65	30.65
Tubificoides sp.	21	9.58	1.96	16.14	46.79
Corophium sp.	17.78	9.26	2.11	15.6	62.4
Alitta succinea	7	4.41	4.88	7.43	69.83
Balanus sp.	9.67	3.9	1.76	6.57	76.4
Main species for control site Average similarity: 56.39					
Species	Average Abundance	Average Similarity	Sim/SD	Contribution %	Cumulative %
Mytilus edulis	52.22	17.51	2.48	31.05	31.05
Corophium sp.	52.33	15.44	2.95	27.39	58.44
Tubificoides sp.	31	8.41	2.16	14.92	73.36

Table 8.3.3. SIMPER analysis of contribution of individual species to the observed dissimilarity between impact and control sites.

Main species Species	Group Impact Average Abundance	Group Control Average Abundance	Average Dissimilarity	Diss/SD	Contribution %	Cumulative %
Corophium sp.	17.78	52.33	9.26	1.77	19.22	19.22
Mytilus edulis	30.56	52.22	7.58	1.62	15.74	34.96
Balanus sp.	9.67	39	7.06	0.77	14.64	49.6
Tubificoides sp.	21	31	5.73	1.12	11.9	61.51
Spiophanes bombyx	7.33	10.67	3.26	1.06	6.78	68.28
Gammarus salinus	9	1.78	2.41	1.26	5	73.28

Impact on eelgrass

Changes in eelgrass cover

Mean eelgrass cover in control (non-dredged) areas was 75.5 ±15.8%, while inside impacted (dredged) areas was 53.0 ±14.0% and (Table 8.3.4). Fishing impact on the eelgrass population estimated from the reduction in eelgrass cover due to dredging was 22.5 ±7.8% (Table 8.3.4).

Table 8.3.4. Diver visual assessment of mean % eelgrass cover in pairwise adjacent quadrats in control areas outside and dredged areas inside mini-dredge tracks. Fishing impact is the mean difference in % in eelgrass cover between control and dredged areas.

		Eelgrass
Area	N	% cover ±SE
Dredged	30	53.0 ±14.0
Control	30	75.5 ±15.8
Efficiency		22.5 ±7.8

Changes in eelgrass root abundance

The mini-dredge caught 84.4 ±19.8 (SE) g/m^2 dredged of eelgrass, of which broken leaves accounted for 53.7 ±9.5 (SE) g/m^2 dredged (Table 8.3.5), with ca. half of those being alive (52.4 ±0.4 %, SE). The number of eelgrass roots caught by the dredge was 11.6 ±3.2 (SE) roots/m^2 dredged (Table 8.3.5). Eelgrass abundance in control non-dredged areas was 469.0 ±51.7 (SE) g/m^2 with a root density of 110.3 ±11.4 (SE) roots/m^2 dredged (Table 8.3.5). Fishing impact on the eelgrass population as % removal of eelgrass roots was 10.5 ±3.1% (Table 8.3.5). If estimated as the % removal of eelgrass biomass, thus closer to cover-based estimates, fishing impact on the eelgrass population was 18.0 ±4.7% (Table 8.3.5).

Table 8.3.5 Eelgrass mini-dredge catches and abundance in a control non-dredged area (g or number/m^2 dredged, ± SE). Fishing impact is the % removal of eelgrass biomass or roots by the mini-dredge relative to control areas.

		Eelgrass		
	N	Biomass (g m^2)	Broken Leaves (g/m^2)	Root density (root/m^2)
Dredge Catch	5	84.4 ±19.8	53.7 ±9.5	11.6 ±3.2
Control	12	469.0 ±51.7	-	110.3 ±11.4
Impact		18.0 ±4.7		10.5 ±3.1

8.4 Discussion

This report presents an evaluation of the fishing efficiency and impact on benthic communities of a purpose-built fishing tool (mini-dredge) with intended use in very shallow conditions by small fishing boats. The rationale is that very shallow habitats cannot be fished by larger boats, while hand-picking from shore often is depth restricted and also on the how much of the biomass that can be removed efficiently. The target habitat for the mini-dredge is thus depths shallower than eelgrass beds and this report evaluates its fishing efficiency and impact in two areas with different Pacific oyster population structure: a shallower high-density population with reef structures and a deeper, lower-density population offshore of the dense population, but shallower than eelgrass beds. Due to the proximity of shallow pacific oyster populations and eelgrass beds, the impact of the mini-dredge on eelgrass was also

assessed for the possibility that dredge tracks or other fishing activities (e.g. dragging of the mini-dredge bag without fishing) would encroach on eelgrass.

Fishing efficiency

The mini-dredge fished efficiently both populations, removing 49% of live Pacific oysters in dense areas and almost twice that at 82% in the offshore population. However, fishing efficiency did not differ between the two populations due to the large variability observed. Fishing efficiency of oysters aggregated into clumps was higher at 100%, but not different than individual non-clumped oysters at 40 and 77%. Dead oyster shells were fished with similar efficiency as live Pacific oysters in both populations, respectively at 40 and 100%. Mini-dredge by-catch was lower in the dense than offshore population, respectively 20 and 61 g/m^2 dredged, consisting mainly of blue mussels and macroalgae attached to live or dead Pacific oyster shells. Other invertebrates constituted a small proportion of by-catch and were higher in the dense population. A single fish (*Gobiidae* sp.) was caught trapped in an oyster shell.

Impact on benthic fauna and macroalgae

The communities found at the dense and offshore populations differed significantly for a range of metrics, which support the assessment of mini-dredge impacts to be done separately for each population. Generally, the dense population was associated with higher abundance, species richness and diversity of epifauna and infauna, and macroalgae abundance. Community composition was also significantly different between dense and offshore populations. The mini-dredge was found to have a significant impact on benthic communities at the dense population, but not at the offshore population. However, for the major large macrofauna only, no differences were observed in the dense population, with only Pacific oyster abundance, the target fishing species, as well as macroalgae abundance being lower in the impacted site.

The differences at the dense population were driven by species from various taxonomic groups: *Corophium* sp., *Balanus* sp., and *Gammarus salinus* (crustacean), *Mytilus edulis* (Bivalve), *Tubificoides* sp. (Oligochate), *Spiophanes bombyx* (Polychaete), and for the major large macrofauna by Pacific oyster and also macroalgae. The lower abundance of *Corophium* sp. and *Mytilus edulis* in the impact areas, as well as of Pacific oysters and macroalgae, might be explained as these are both sessile species, which live in (*Corophium*, Pacific oyster and macroalgae) or on (*M. edulis*) the sediment. These would therefore be vulnerable to the passing of the mini-dredge. By contrast, *Gammarus* sp. (found in higher abundance in the impact area) are relatively mobile and may be redistributed/swim into the water column during the passing of the min-dredge.

At the offshore area, there were no significant differences in abundance, species richness, diversity, and community composition between control and impact sites. This suggests no effect of fishing at the offshore population. However, communities were generally less abundant and species richness lower in the impact site at the offshore population. The generally poorer communities recorded at the offshore population may indicate the possible effect of some other pressure (compared to the dense population, e.g. higher depth or the absence of Pacific oyster reefs), which may have affected the ability to detect an impact from mini-dredge fishing.

Overall, the results suggest mini-dredge fishing at the dense population resulted in lower abundance, species richness and diversity, and altered community composition than at the control site. However, due to the design of the study with no information before fishing impact, it cannot be discounted that communities in the control and impact sites were inherently different, regardless of the effect of fish-

ing, even though less than 25m separated control and impact sites. The study assessed the maximum impact expected from mini-dredge fishing as by-catch was landed. Under fishing conditions, the by-catch is discarded back to the local habitat and a proportion, particularly the more resilient fauna, are expected to survive and thus the impact will be lower than the one observed. Macroalgae not attached to shells or stones are not expected to benefit from discard back to the local habitat, since macroalgae cannot reattach once removed from oysters or stones and thus will not survive, whereas attached macroalgae will have the potential to survive. In addition, Pacific oyster reefs can positively provide impacts on diversity, community structure and ecosystem functioning, which vary with habitat, environmental conditions, location and oyster density (e.g. Padilla 2010; Troost 2010; Laugen et al., 2015; Herbert et al., 2016). Therefore, it cannot be discounted that at least a fraction of the observed mini-dredge fishing impact on the dense population, which contains reef structures, resulted from the removal of the reef structure impacting associated fauna, and not from a direct fishing impact. If so, whatever method used for removal of the invasive Pacific oyster reef structures, including low impact hand-picking, will have an impact on the reef associated benthic community and its functions. However, an assessment would be required to determine what are the effects and if Pacific oyster reefs affect negatively or positively local diversity, community structure and ecosystem functioning services in such microtidal and very shallow habitat as in the Limfjorden.

Impact on eelgrass

Mini-dredge fishing was found to significantly impact eelgrass populations, and any fishing with the mini-dredge must avoid areas with eelgrass. Fishing resulted in the reduction of 22% in cover, 18% in biomass and 10.5% in eelgrass root density. These impacts were due to single fishing events, which implies that multiple fishing events at the same location (i.e. repeated overlapping dredge tracks) will increase total impact.

8.5 Conclusions

The new mini-dredge tool for the fishing of Pacific oyster populations in very shallow habitats was found to be efficient in the capture of the target species. However, even with precautionary measures incorporated into its design – i.e. light weight, side skids to avoid digging into the sediment and short teeth to only catch large objects sitting on and not within the sediment – mini-dredge fishing had a significant impact on the benthic community of Pacific oyster populations. Such impact can be partially mitigated by the return of the by-catch to the local habitat but could also be perceived as a return to native conditions by removal of an invasive species. In the event mini-dredge fishing activities extend into eelgrass populations, a significant impact will occur, and any fishing must ensure areas containing eelgrass are avoided. Furthermore, the location of the Pacific oysters in very shallow areas (<1.5 m) makes the fishery with the mini-dredge from a boat very unpredictable as the areas can only be accessed at high water levels, which in a microtidal areas (tidal amplitude <30 cm) only occur a few days/weeks per year.

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Mitigation tool 2: Removal efficiency and impacts of a floating excavator for removal of very shallow (<1.5 m), dense Pacific oyster populations in the Limfjorden

Authors: Pedro S. Freitas, Patrick Joyce and Pernille Nielsen

8.7 Introduction

Under certain conditions, mitigation measures of local impacts from dense Pacific oyster populations may be required its removal to ensure the use and access to and from the coast for water-based activities (e.g., bathing, kayaking, fishing). Floating excavators may provide a fast and efficiency tool to remove Pacific oysters, which require assessment of its removal efficiency and impacts on shallow communities and habitat.

Within this study, a floating excavator was hired to remove dense populations of Pacific oysters to evaluate and analyse the efficiency and impact. The study had the following aim:

> Determine the efficiency of a floating excavator for the removal of Pacific oysters from dense populations in shallow habitats (<1.5m), as well as assess impacts on shallow macrofauna and macroalgae and its persistence after 2 years.

Approach
Field trials were carried on a dense Pacific oyster population (Figure 8.8.1), where a floating excavator commonly used in rivers, lake shores and wetlands was used to fully remove patches of Pacific oysters (Figure 8.8.2).

8.8 Methods

Trial site
The trial site was a large Pacific oyster bed located at Klosterbugten Mors in the Limfjorden Denmark (Figure 8.8.1), which is a small bay next to the town of Nykøbing Mors. The Pacific oyster bed sits next to a marina in an area used by the public for water-based activities, e.g. swimming, paddle boarding, etc. The Klosterbugten Pacific oyster bed has been surveyed several times since 2006 (Groslier et al., 2014, Section 2 this report), and has expanded considerably since 2010, but suffered significant winter mortality events in 2018 and 2020 (Section 2 this report, unpublished observation). Macroalgae cover in Klosterbugten and on the Pacific oyster bed is high, particularly in dense-reef areas that now contain a large fraction of dead oysters (unpublished observation).

Excavator
An excavator E10 REMU commonly used for clearance work in river and lake shores and wetlands was rented from Sloths Naturpleje company (Figure 8.8.2). The excavator weighed 8 tons and was mounted on floating tracks with a metal mesh 3m by 1m bucket.

Trial design
A control-impact design was used with impacted plots having Pacific oysters removed by the excavator and nearby undisturbed areas used as control sites (Figure 8.8.1). Three different oyster density areas identified by previous surveys (Section 2 this report) were used (Figure 8.8.1): a low-density mainly with isolated oysters, a medium-density with isolated and some clumped oysters and a high density reef area.

On impact plots (3 x 1m, N = 3 per density), all Pacific oysters present were removed by scraping the bottom with the excavator in May 2020 (Figures 8.8.1 and 8.8.3). Nearby control sites were sampled using quadrats (25 x 25 cm, N = 8 per density). A follow-on assessment was done 22 months after the excavator removal action in April 2022 with quadrats sampling (25 x 25 cm) in each of the impact plots and control areas in (Figure 8.8.1, N = 6 per density). Macrofauna and macroalgae abundance were determined in both impacted and control sites.

Community composition was described as abundance (number or weight of individuals), species richness (number of species), Shannon's diversity (H) and evenness (E_H) indices (Table 3 Appendix 8.2) : $H = -\Sigma(p_i * \ln p_i)$, where Σ is the sum of the natural log of pi, the proportion of the entire community made up of species i; $E_H = H / \ln (S)$ where, H is the Shannon Diversity Index and S is the total number of unique species. E_H ranges from 0 to 1 and 1 indicates complete evenness. It must be noted that highly mobile fauna, e.g. fish, shrimps and crabs are mobile and likely avoided sampling. Excavator impact was assessed as changes in macrofauna species richness, abundance and community composition between control and impact sites using multivariate analysis. Data were log transformed, and resemblance matrices calculated using Bray-Curtis similarities. Cluster analysis, non-metric multidimensional scaling (NMDS) and multivariate Anova (Permanova) were carried to test if community composition (or species composition) of differed between control and impact sites separately for dense and offshore populations. SIMPER (similarity percentage) analysis was done to identify which species made the greatest contribution to observed dissimilarity between groups.

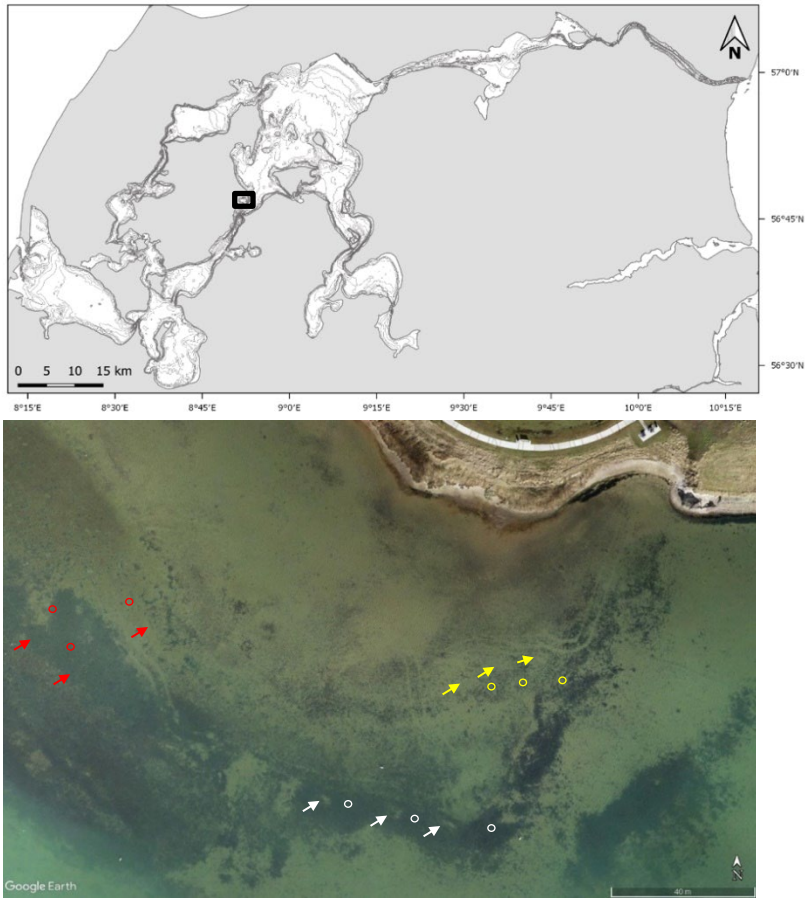

Figure 8.8.1. Location of trial site in Klosterbugten on the Island of Mors, Limfjorden (Top, black rectangle). 3 impact plots (3m by 1m) were excavated in each density area: Low (red arrows), medium (yellow arrows) and high (white arrows) densities. The latter corresponded to oyster reefs. Circles indicate control sites sampled with quadrats. Overlaid on a high-resolution drone image mosaic obtained in March 2022 (Aris Thomasberger, DTU Aqua), 22 months after impact dredging in May 2020 (Google Earth).

Figure 8.8.2. The E10 floating excavator (left) and sampling during the field trial in Klosterbugten in Mors, Limfjorden (right) (Photos: Sloths Naturpleje (left) and Pernille Nielsen, DTU Aqua).

Figure 8.8.3. 22 months after excavation impact: Excavated plots are the bare patches seen in high (left image, centre) and medium (right image, centre-right) density areas., surrounded by non-impacted areas with more abundant oyster and macroalgae cover (Photos: DTU Aqua).

8.9 Results

Excavator Catches

The excavator removed between 0.41 and 7.3 kg/m^2 of live Pacific oysters, corresponding to a catch between 2.4 and 28.3 oysters/m^2 (Figure 8.9.1 and Tables 1 and 2 appendix 8.2). Although mean catch of live Pacific oysters in high density area was over 10 times than in low and medium density areas, the difference was not significant (non-parametric Kruskal Wallis, $p > 0.05$). Only the catch of dead Pacific oysters was significantly higher in the high density than the low-density areas (non-parametric Kruskal Wallis and Dunn pairwise tests, $p < 0.05$).

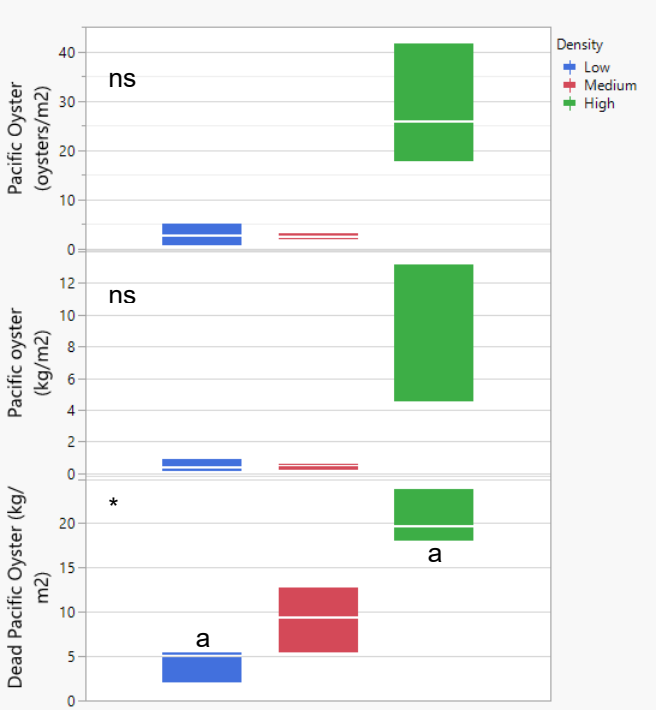

Figure 8.9.1. Live pacific oyster density (oysters/m^2) and biomass (kg/m^2) and dead Pacific oyster catch (kg/m^2) in the low (blue), medium (red) and high (green) density areas. Asterisk indicates level of significance in differences, while letter indicates areas that are different (non-parametric Kruskal Wallis and Dunn pairwise tests): * <0.001, ** <0.01, * <0.05, ns. – not significant.**

Total mean catches of the excavator were significantly higher in the high than the low-density areas (KW and Dunn tests, $p < 0.05$) and ranged between 9.4, 33.9 and 57.1 kg/m2 respectively in the low, medium and high-density areas (Figure 8.9.2 and Table 1 appendix 8.2). Dead shells and stones were the main component of the excavator catch in the three density areas and ranged respectively between 2.3 and 50.7 kg/m^2 and 2.3 and 0.1 and 15.4 kg/m^2 (Figure 8.9.2 and Table 1 appendix 8.2). Despite such range, no significant difference occurred between the three density areas (KW test, $p > 0.05$). By-catch of live organisms was significantly higher in the high than the low-density areas (KW and Dunn tests, $p < 0.05$). Mean by-catch ranged between 40, 1,565 and 4,318 g/m^2 in respectively the low, medium and high-density areas, corresponding to 3.8, 34.1 and 160 individuals/m^2 (Figure 8.9.2 and Table 1 appendix 8.2).

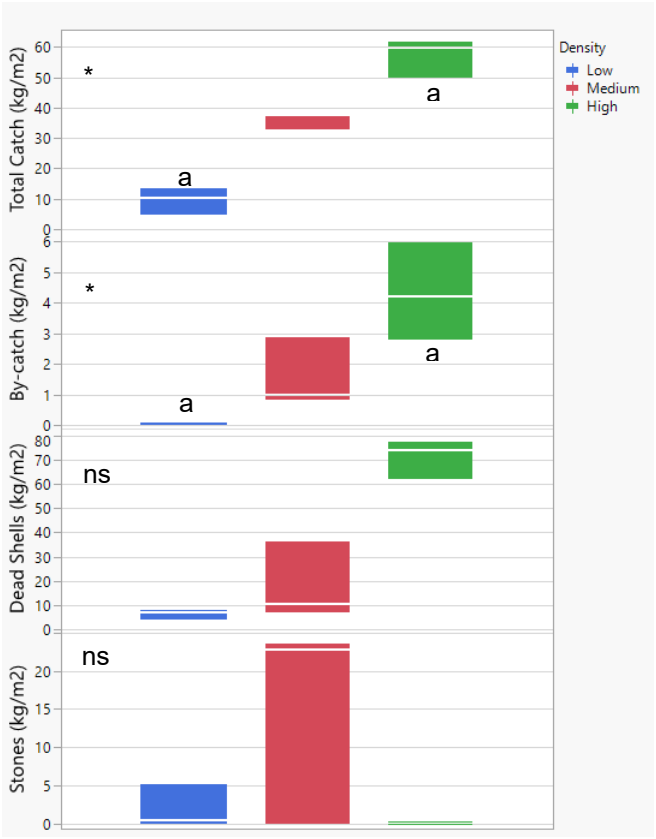

Figure 8.9.2. Total weight, live by-catch, dead shells and stones caught by the excavator (kg/m^2) in the low (blue), medium (red) and high (green) density areas. Asterisk indicates level of significance in differences, while letter indicates areas that are different (non-parametric Kruskal Wallis and Dunn pairwise tests): * <0.001, ** <0.01, * <0.05, ns. – not significant.**

Live by-catch, excluding macroalgae which made the majority of by-catch by weight (Figure 8.9.3), was made of 2 species of fish, the sand goby *Pomatoschistus minutus* in 7 out of the 9 excavated samples and in a single occasion, the eel pout *Zoarces viviparus*; three species of bivalves, the blue mussel *Mytilus edulis* present in all samples, and the common cockle *Cerastoderma edule* and grooved carpet clam *Ruditapes decussatus* in a single sample. Other invertebrate species included the shore crab *Carcinus maenas* and the gastropod *Littorina littorea* in 7 samples; less common were the gastropod *Nassarius reticulata* and *Crepidula fornicata*, the shrimps *Palaemon serratus* or *Crangon crangon*, the anemone *Metridium senile* present in less than 3 samples. Unidentified polychaete worms were present in 5 samples.

Assessment of the catches by the industry

The sorted alive oysters (individual and clumps) were split in two fractions, one for each of two local processing industries, which made an assessment if the oysters removed by the excavator was of commercial interest.

Company 1 tried to open a few of the oysters by either microwaves or with oyster knifes, which was very difficult. The visual assessment of the catches showed that the oysters were too big (e.g., some >600 g), often had a skewed shape and with a lot of macroalgae attached. The overall conclusion was that there is no market for these types of oysters. Company 2 sorted the oysters into different size categories and assessed the average meat content to be 12-13% and generally no damages of the shells were observed. Approximately 35% of the catch was oyster of 150-200 g, which can be solid for the fresh market at a prize of 20-25 DKK per kilo, 17% were 200-300 g oysters that potentially

could be sold at a lower price and the rest (47%) were >300 g and currently there is no market for them. All oysters needed to be cleaned for biofouling before they could be sold.

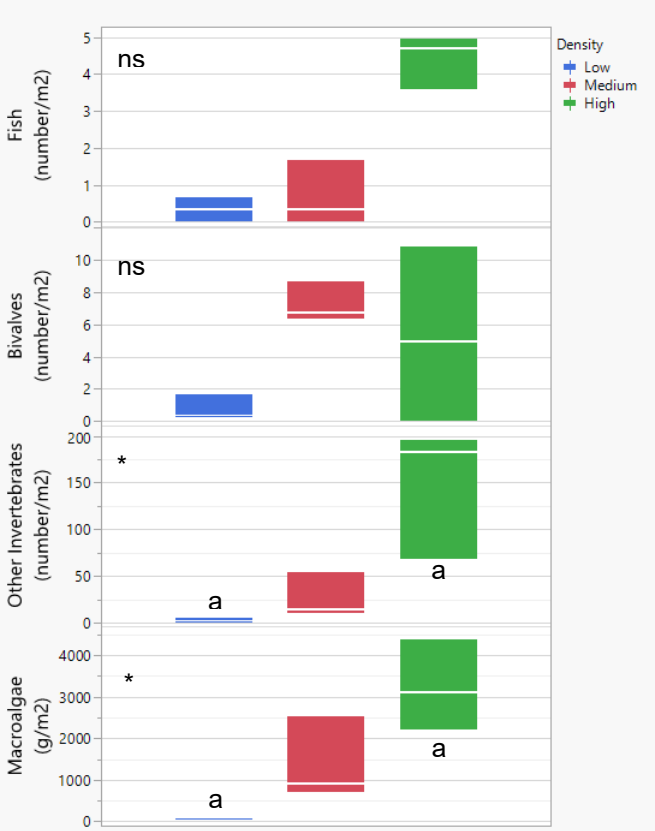

Figure 8.9.3. By-catch: Fish, bivalves and other invertebrates density (individuals/m^2) and macroalgae biomass (kg/m^2) caught by the excavator in the low (blue), medium (red) and high (green) density areas. Asterisk indicates level of significance in differences, while letter indicates areas that are different (non-parametric Kruskal Wallis and Dunn pairwise tests): * <0.001, ** <0.01, * <0.05, ns. – not significant.**

Community composition differences with density

Macroalgae abundance at control sites was similar in different density areas (KW test, $p > 0.05$), except between high- and low-density areas before impact (Dunn test, $p = 0.018$). Nevertheless, PERMANOVA tests showed macroalgae community composition in control sites to be different in different density areas both before and after impact ($p = 0.0084$ and $p < 0.0001$, respectively). Macrofauna species richness, abundance and diversity at control sites were not significantly different in the three density areas before the excavator impact (KW test, $p < 0.05$), but were significantly different 22 months after the excavator impact (Figure 8.9.4, KW test, $p < 0.05$). PERMANOVA tests confirmed the existence of significant differences in macrofauna community composition at the three density sites 22 months after impact ($p = 0.0013$), but not before ($p > 0.05$). Therefore, excavator impact on macrofauna and macroalgae needs to be analysed separately for each density area.

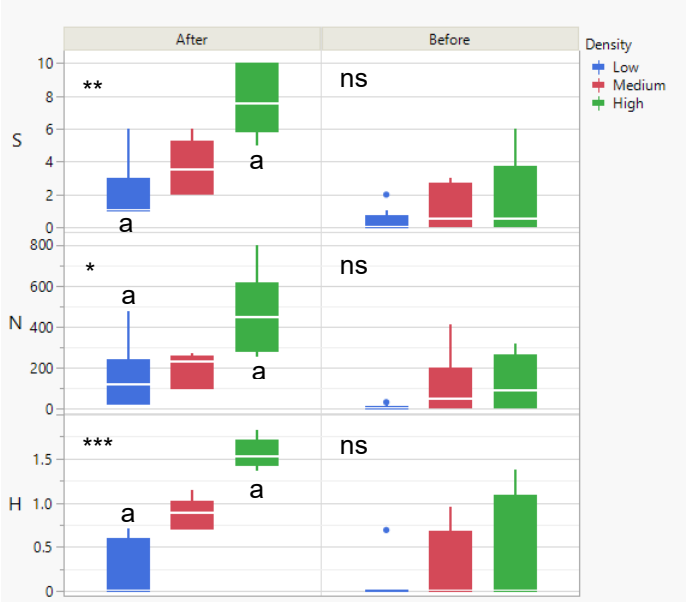

Figure 8.9.4. Species richness (S), abundance (N) and Shannon's diversity index (H) in control sites in the low (blue), medium (red) and high (green) density areas. Asterisk indicates level of significance in differences (non-parametric Kruskal Wallis and Dunn pairwise tests): * <0.001, ** <0.01, * <0.05, ns. – not significant.**

Non-impact related temporal evolution of control sites

Macroalgae abundance was similar before and after impact in all three density areas (KW test, p > 0.05). Macroalgae community composition, however, was different before and after excavator impact in all three areas (PERMANOVA, p >0.05).

Macrofauna species richness, abundance and diversity at control sites were generally significantly higher after than before impact (Figure 8.9.4, KW test, p >0.05), except abundance at medium density area and diversity in low diversity area (Figure 8.9.4, KW test, p >0.05). A total of 6 species were identified before impact, while 16 species were identified after impact. Total abundance was higher after impact (N = 2800, 1184, and 912, respectively) than before impact (N = 992, 848 and 48, respectively, Table 4 appendix 8.2). PERMANOVA tests confirmed the significant temporal differences before and after excavator impact in macrofauna community composition at control sites in the three density areas (p = 0.0154, p = 0.0165 and p = 0.0009, respectively). SIMPER analyses (Table 6 appendix 8.2) showed that in high and medium density areas polychaete worms, the bivalves *M. edulis* and *C. gigas*, and the gastropod *L. littorea*, accounted for most of the dissimilarity between before and after impact (55% and 79%, respectively). While in low density areas, amphipods accounted for 48% most of the dissimilarity, with the four taxa mentioned for high and medium densities accounting for further another 48%. In all cases, abundance was higher after impact, except for the Pacific oyster in low density areas, which was lower after impact.

In summary, a significant temporal change in macrofauna and macroalgae community composition occurred at control sites that was independent of excavator impact: the macroalgae and macrofauna communities were different; macrofauna species richness, abundance, and diversity increased from the time of impact to 22 months afterwards. The Pacific oyster, which was the target of removal by the excavator, increased in abundance at undisturbed control sites in high and medium density areas and decreased in low density areas from the time of impact to 22 months afterwards.

Excavator impact after 22 months: Impact versus control sites

After 22 months, the abundance of Pacific oysters was significantly higher at control than at impact sites (KW test, p = 0.019 and p = 0.0052, respectively) in high and medium density areas: 61.33 ±44.90 SE oysters/m^2 in control and 5.33 ±8.26 SE oysters/m^2 in impact sites, while in medium density areas it was 10.67 ±8.26 SE oysters/m^2 in control and 0 oysters/m^2 in impact sites. Pacific oyster abundance was zero at both control and impact sites in low density areas.

Five macroalgae species were identified in both control and impact sites. Macroalgae abundance was similar in high- and low-density control and impact sites (KW test, p < 0.05), but higher in medium density control sites (KW test, p = 0.0033). Macroalgae community composition was different in high and medium density areas control and impact sites, but not in the low-density area (PERMANOVA, p = 0.0193, p = 0.0105 and p = 0.2564, respectively). A total of 16 macrofauna species were identified in control sites and 12 species in impact sites (Table 5 in appendix 8.2). Total abundance was higher at high and medium density control sites (N = 2800 and 1184) than at impact sites (N = 768 and 400), but the reverse occurred in low density areas due to the high abundance of amphipods (N = 1872) that accounted for 80% of abundance in impact sites (N = 2336) but only 56% (N = 512) in control sites (N = 912).

Figure 8.9.5. Species richness (S), abundance (N) and Shannon's diversity index (H) in control (blue) and impact sites (red) in the low, medium and high density areas. Asterisk indicates level of significance in differences (non-parametric Kruskal Wallis and Dunn pairwise tests): * <0.001, ** <0.01, * <0.05, ns. – not significant.**

Macrofauna species richness, abundance and diversity were significantly higher at high and medium density control than impact sites (Figure 8.9.5, KW test, p < 0.05, table 4 appendix 8.2), but were not different at low density (Figure 8.9.5, KW test, p > 0.05). Macrofauna community composition was significantly different at high and medium density control and impact sites, but not in the low-density area (PERMANOVA, p = 0.0021, p = 0.0062 and p = 0.6835, respectively). SIMPER analyses (Table 7 appendix 8.2) showed that most of the dissimilarity between high and medium density control and impact sites (51% and 77%, respectively), were accounted for by polychaete worms, the bivalves *M. edulis* and *C. gigas*, and the gastropod *L. littorea*. While in low density areas amphipods accounted for 32% of the dissimilarity between control and impact sites, with the gastropod *L. littorea*, polychaete worms and the sand goby *Pomatoschistus minutus* accounting for a further 19%, 12% and 12%, respectively. The abundance of most species was generally higher at control than impact sites in high

and medium densities. Except the gastropod *N. reticulata*, the bivalve *C. edule* and isopoda sp., which were more abundant at impact sites, but each accounted for less than 7 % of dissimilarity. In the low-density area, the reverse was observed with higher abundance at impact sites (Table 7 appendix 8.2). Pacific oysters were absent at impact sites except in the high-density area.

8.10 Discussion

This report presents an evaluation of Pacific oyster removal efficiency of a floating excavator commonly used in clearance work in wetlands, river and lake shores, as well as its impacts on benthic communities from removal activities. Pacific oyster removal and impacts by the excavator were assessed in a high-density, reef containing, population over a range of densities.

The floating excavator was highly efficient in the removal of Pacific oysters, with 100% removal efficiency at low and medium densities and 91% at high density remaining after 22 months. Since the excavator fully scraped the bottom, the lower removal efficiency at high densities likely originated from bordering dense reefs structures becoming unstable from excavation and oysters eventually falling into excavated plots. Overall significant, long-lasting impacts in both macroalgae and macrofauna communities were observed 22 months after to excavator removal activities.

Macroalgae and macrofauna had lower abundance, species richness and diversity, and altered community composition in excavated impacted sites than in non-excavated control sites, except at low density areas where no differences were observed. Polychaete worms, the blue mussel bivalve *M edulis* and the pacific oysters, and the gastropod periwinkle *L. littorea*, all had low abundance in impacted sites except in low density areas, where several taxa (e.g. amphipods, worms, isopods) were more abundant in impacted areas. Impacts were long-lasting after 22 months, suggesting a lasting effect on the settlement/recruitment of sessile species, such as bivalves (i.e. blue mussels and Pacific oysters), but also a likely change in habitat from excavation impacts that disfavour mobile species, such as gastropods, amphipods, shrimps and fish at medium and high Pacific oyster populations, but not at low density populations. Populations of the invasive Pacific oyster can impact and increase the diversity, community structure and ecosystem functioning, which vary with habitat, environmental conditions, location and oyster density (e.g. Padilla 2010; Troost 2010; Laugen et al., 2015; Herbert et al., 2016). The observed changes in benthic community abundance, diversity and composition suggest, but require further confirmation, that populations of the invasive Pacific oyster likely had an increasing effect at medium to high densities, where Pacific oyster can form non-native biogenic reefs or dense populations, but a decreasing effect at low density. However, the establishment of Pacific oyster non-native biogenic reefs and dense populations in the very shallow areas of the Limfjorden change the natural habitat by creating biogenic reef and hard-organism habitats that are otherwise typically absent in these areas.

Temporal changes in abundance and community composition unrelated to excavation activities were observed between the time of impact and the follow-on assessment 22 months afterwards. Such changes increased macrofauna species richness, abundance, and diversity, as well as Pacific oyster abundance, and thus favoured the recovery of impact sites after removal activities. Due to the study design, it cannot be discarded that macroalgae and macrofauna communities at the time of excavation were inherently different between control and impact sites but based on the spatial variability observed between control sites such differences are assumed to be relatively small and clearly not to the extent of the differences observed 22 months after excavation. Nevertheless, it cannot be discarded that temporal changes and pre-impact differences between control and impact sites may have biased, either amplifying or diminishing, the observed impacts from excavator removal activities. Several of the impact plots could still be identified by eye or by a change in bathymetry 22 months after

excavations activities (Figures 8.8.1 and 8.8.3), e.g. in the high density reef area impact plots can easily be identified in drone images as bare light patches surrounded by oyster reef and macroalgae, which in the field corresponded to a distinct step like drop in bathymetry of 30 cm (Figure 8.8.1, tips of white arrows and Figure 8.8.3) or in mid density impact plots as bare light patches (Figure 8.8.1, tips of yellow arrows and Figure 8.8.3). Noticeably, even the marks left at the bottom by most of the floating excavator tracks when moving to execute the field trial are still visible in drone images (Figure 8.8.1).

8.11 Conclusions

The floating excavator was highly efficient (100-91%) in the removal of invasive Pacific oysters, at low, medium and high densities. However, the long-lasting impacts in both macroalgae and macrofauna communities were observed 22 months after removal activities. Furthermore, several of the impact plots could still be identified by eye or by a change in bathymetry 22 months after excavations activities and most of the marks left at the bottom by the floating excavator were still visible in drone images. Such impact of the tracks of the floating excavator can be partially mitigated by carrying out the experiment at a higher water level to make the excavator float, whereas alterations in the impact plots cannot be avoided.

8.12 References

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Mitigation tool 3: Development and testing of on-board sorting equipment for mixed catches of blue mussels and pacific oysters

Authors: Pernille Nielsen and Pedro S. Freitas

The occurrence of mixed beds or reefs of Pacific oysters and blue mussels can be an obstacle initiating commercial mitigation of the pacific oysters. This can be due to that only low percentages or no bycatches of blue mussels are allowed. The fishers have therefore shown an interest in techniques to separate the two species allowing them to subsequently relay blue mussels while the Pacific oysters are landed. The incentive to mitigate the invasive Pacific oyster is then driven by the commercial utilisation of the Pacific oyster and/or to preserve blue mussel beds from potentially turning into oyster reefs, which has less economic value for the fishers.

8.13 Materials and methods

Description of the sorting equipment

A new sorting equipment installed onboard the fishing vessel Wilhelmina (E78) was developed and tested by Fiskeriselskabet Cardium A/S. The sorting equipment consists of two large (2.5 x 2.7 m) sorting tables, each with a hydraulic washer connected (Figure 8.13.1, left). The catch is flushed down through the slide door when open manually and transported by the conveyor belt up to the sorting drum (Figure 8.13.1, right). After the installation of the sorting equipment on-board the vessel the equipment was tested on the 16th of October 2019 by Fiskeriselskabet Cardium A/S.

Figure 8.13.1. Left: the inside of one of the sorting tables with the two sliding doors in each corner and the hydraulic washer outlet in the centre. Right: Sorting drum with the conveyer belt at the lower left corner (Photos: Esbjerg Shipyard).

Selection of area and description of the beds

Previous surveys of blue mussels and Pacific oyster have shown that in the areas north of Fanø and in Ho Bugt mixed beds of blue mussels with individual Pacific oysters were observed (Nielsen et al. 2019). Consequently, two mixed mussel-oyster beds south of Langli in Ho Bugt were selected for carrying out the experiments of the sorting equipment (Figure 8.13.2). A visual inspection of the beds was done before any sampling took place. The length of each of the beds were approximately 100 m, 25-50 m in width and approximately 50 cm high. The two beds seemed similar and could be characterised as blue mussel beds with individual Pacific oysters of typically <10 cm with either high densities or full coverage of blue mussels, where some of part of the beds were covered by macro algae (Figure 8.13.3).

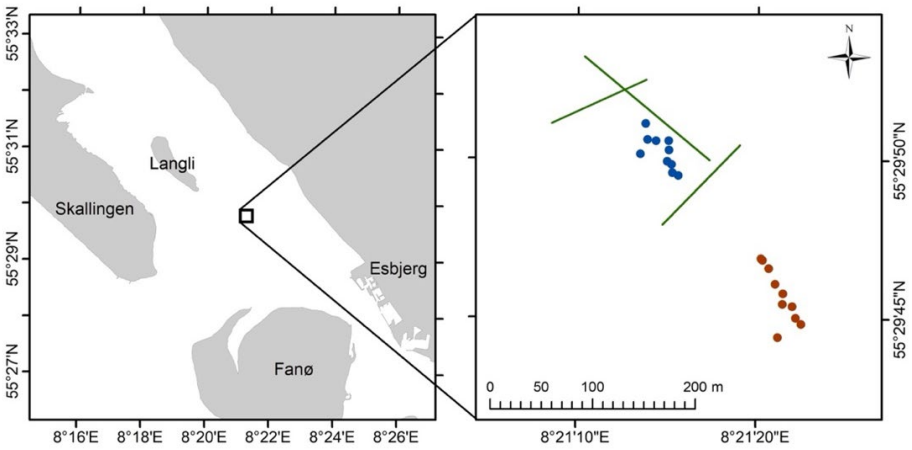

Figure 8.13.2. Left: Map of the location of the two selected mixed mussel beds were located. Right: Positions of the ten frame samples at either the non-fished area (red dots) and the fished area (blue dots) and the positions of the three dredge tracks (green lines) carried out during the testing of the sorting equipment in November 2021.

Assessment of recolonisation of Pacific oysters in a fished area

Before the fishing activity was carried out, ten quadrates (0.25 x 0.25 m) were collected along the entire length of each bed with approximately ten meters between each quadrate. For each quadrate, the position was determined by marking the GPS position of the sampling location. Within each of the quadrates, the top layer of approximately ten centimetres of the bed, where the alive species are observed, was collected. The samples were weighed to determine total wet weight (WW) and the catch was subsequently sorted and weighed, separating Pacific oysters, blue mussels, empty shells, stones, other epifauna and macroalgae. Furthermore, shell length was measured to the nearest 0.5 cm for all oysters and blue mussels (or a maximum of 200 individuals) at each station.

Figure 8.13.3. Quadrate sampling at one of the mussel beds south of Langli in November 2021. Individual Pacific oysters are evenly distributed across the entire beds, whereas the cover of macro algae is patchier and often observed at the edges (Photo: Pernille Nielsen).

A follow-up study of the recolonisation of the fished areas was not carried out within the project due to an extended processing period (~2.5 years) for getting the permit from the authorities. Consequently,

the testing of the sorting equipment could not take place before November 2021 and with the projected ending in April 2022, a follow-up study less than a half a year after fishing and before a new spawning season would not provide sufficient information about recolonisation of the fished area.

Fishing event and testing the sorting equipment

The scientific fishery testing was carried out with a commercial fishing vessel in November 2021. The fishing took place with a "Limfjordsskraber" a modified Dutch dredge (width: 2 m and total maximum weight: 250 kg) and with a wire length of <100 m at the port side of the vessel. Three dredge tracks were carried out at a towing speed of 3-4 knots. The length of three dredge tracks varied from 100-162 m resulting in a total catch of 210 kg of blue mussels and 150 kg of Pacific oysters, impacting an area of at 744 m^2. The catch was unloaded into the sorting tables and passed through the sorting drum separating the blue mussels as a relatively clean fraction, whereas the oyster fraction contained any larger fragments e.g., stones, oysters, shells, clumps of macroalgae (Figure 8.13.4).

Figure 8.13.4. Left: Sorting drum showing that the separation of the blue mussels occurs at the start of the drum and Pacific oysters and other larger parts are transported to the end of the drum. Right: Sorted clean fraction of blue mussels (Photo: Fiskeriselskabet Cardium A/S).

Assessment of the efficiency of the sorting equipment

From the two sorted fractions, approximately half of the total catch was examined further by collecting nine and six random selected subsamples from the blue mussel and Pacific oyster fractions, respectively. Each subsample was weighed to determine total wet weight (WW) and each fraction was then subsequently sorted and weighed, separating Pacific oysters, blue mussels, empty shells, stones, other epifauna and macroalgae to determine the bycatch in each fraction and numbers of damaged blue mussels or Pacific oysters, Furthermore, shell length of Pacific oysters and of blue mussels were measured to the nearest 0.5 cm for all oysters and blue mussels (or a maximum of 200 individuals) per subsample.

8.14 Results and discussion

Sorting efficiency and bycatch of blue mussels and Pacific oysters

The sorting equipment sorted the two species into two separate fractions and with a low percentage of 0.05% of damaged/broken blue mussels in each of the two fractions, whereas a few Pacific oysters had minor damages of the edges of the shells (Figure 8.14.1). Blue mussels constituted 51% of the mussel fraction, whereas the Pacific oysters constituted 30% of the oyster fraction, reflecting the

cleaner blue mussel fraction compared to the Pacific oyster fraction (more details below). In the Pacific oyster fraction, the blue mussels accounted for 19% of the total weight, as the mussels were attached to the oysters. A commercial fishery of Pacific oysters with dredges will result in bycatch of blue mussel no matter if the fishery is targeting mussel beds mixed with Pacific oysters or oyster reefs with associated blue mussels. Consequently, the bycatch of mussels cannot be avoided but could potentially be reduced, if the fishery takes place during summer, as the attachment of blue mussels is relatively strong during autumn and winter compared to summer (Newcomb et al. 2019).

The mussel fraction had a percentage bycatch of pacific oysters of 2.3% (Table 8.14.1), which consisted of Pacific oysters with a shell length <8 cm (data now shown). The relative low bycatch of smaller pacific oysters, provide the possibility for cleaning areas with mixed mussel beds with Pacific oysters by fishing the beds, using the sorting equipment, and subsequently relay a blue mussel bed almost free or only colonised by smaller individuals of Pacific oysters until potential re-colonisation of Pacific oysters occur.

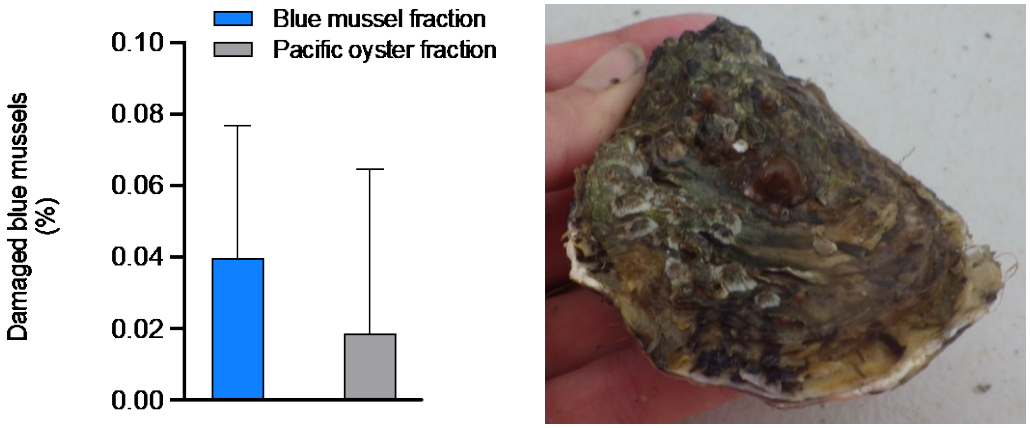

Figure 8.14.1. Left: Graph showing mean percentage (± standard deviation) of damaged blue mussels in either the blue mussel fraction (blue bar) or the Pacific oyster fraction (grey bar). Right: Picture of the observed damages of the edges of the Pacific oyster shells (Photo: Pernille Nielsen).

The shell length size distribution of blue mussels is dominated by smaller mussels in the oyster fraction compared to the mussel fraction (Figure 8.14.2), which is supported by visual inspection during the sorting of the samples, where often multiple smaller mussels were observed attached to both alive oysters and dead oyster shells.

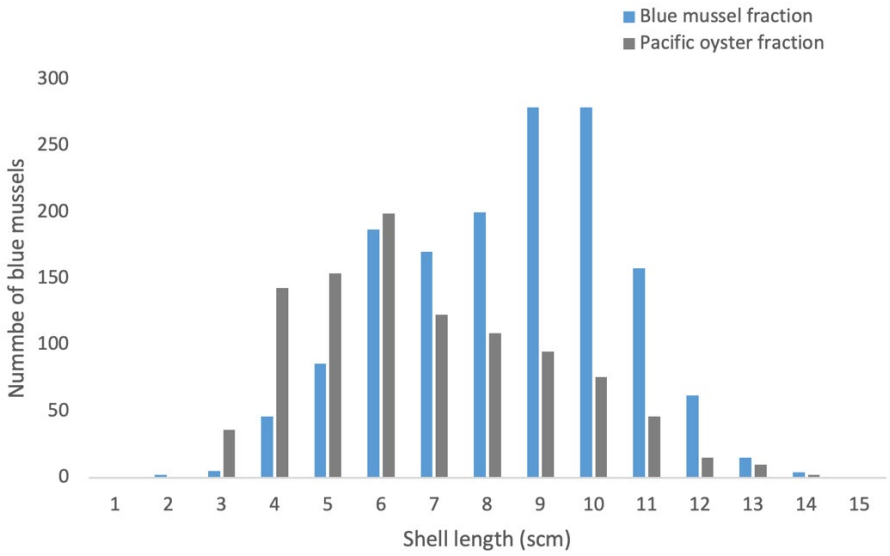

Figure 8.14.2. The number of mussels of different shell lengths in either the blue mussel fraction (blue bars) or the Pacific oyster fraction (grey bars). Scm = semi-centimetres.

Bycatch of other species

The bycatch species in the mussel and oyster fractions were dominated by the same species e.g., common cockle, shore crab, Asian shore crab and the macroalgae toothed wrack, whereas green sea urchin, gobies and worms only were observed in the mussel fraction (Table 8.14.1). In both fractions, the mean bycatch percentages were generally below 1% for other species than blue mussel, pacific oyster, and toothed wrack. The toothed wrack had the highest bycatch 8.1 and 27.6% in the mussel and oyster fraction, respectively (Table 8.14.1). The total mean bycatch percentage for all species was three times higher in the oyster fraction (28.9%) than the blue mussel fraction (9.5%), which is caused by 1) bycatch of blue mussel and 2) a higher bycatch of toothed wrack. The design of the sorting drum causes any larger parts to be transported to the end of the drum and will therefore end up in the oyster fraction. Consequently, the bycatch percentage of macroalgae should be calculated based on both fractions to give a total mean percentage of macroalgae bycatch of 12.3% (Table 8.14.1).

Table 8.14.1. Mean percentage (± standard deviation) bycatch of each species within the subsamples in either the sorted blue mussel fraction (n=9), sorted Pacific oyster fraction (n=6) or for in both fractions (total). – indicate data not included. * Indicate invasive species.

Common species name (*Latin name*)	Mussel fraction % bycatch ± std	Oyster fraction % by-catch ± std	Total % bycatch
Fauna			
Blue mussel (*Mytilus edulis*)	-	19.2 ± 9.2	-
Pacific oyster (*Magallana gigas*)*	2.3 ± 2.2	-	-
Cockles (*Cerastoderma edule*)	0.3 ± 0.3	0.4 ± 0.4	0.2
Shore crab (*Carcinus maenas*)	0.9 ± 0.8	0.8 ± 0.8	0.6
Asian shore crab (H*emigrapsus sanguineus*)*	0.08 ± 0.07	0.02 ± 0.03	0.04
Green sea urchin (*Strongylocentrotus droebachiensis*)	0.02 ± 0.05	0.0 ± 0.0	0.004
Gobies (*Pomatoschistus* sp.)	0.02 ± 0.05	0.0 ± 0.0	0.01
Worms (not identified)	0.002 ± 0.005	0.0 ± 0.0	0.0004
Macroalgae			
Toothed wrack (*Fucus serratus*)	8.1 ± 3.3	27.6 ± 11.8	12.3
Average all species	9.5 ± 3.5	28.9 ± 12.5	13.2

8.15 Conclusion sorting equipment

The sorting equipment was very successful in sorting the two species into separate fractions and with low percentage of damaged/broken blue mussels and Pacific oysters. The bycatch of mussels was relatively high (19%) in the oyster fraction, as often smaller mussels were attached to the oysters. By-catch of mussels cannot be avoided but can potentially be reduced, if the fishery takes place during summertime, when the attachment of mussels is weaker. The relative low bycatch (2.3%) of smaller oysters in the mussel fraction provides the possibility for relay a blue mussel bed almost free of Pacific oysters. Mean bycatch percentages of other species than blue mussel and Pacific oyster were generally <1%, except for the seaweed toothed wrack, which due the attached to the bivalves had an average bycatch of 12.3%.

8.16 References

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9. Testing High Pressure Processing (HPP) technology for opening non-commercial sized Pacific oysters and pilot studies of potential new products

Authors: Pernille Nielsen and Pedro S. Freitas

Wild populations of Pacific oysters are often characterised by heterogenous sizes and potentially also oyster clumps of varying sizes. Since Pacific oyster is an invasive species, it is mandatory for the fishers to land all oysters caught. Consequently, oysters of non-commercial sizes for the fresh market can become an obstacle for developing a sustainable and economic viable Pacific oyster fishery in Denmark as they become a discard and waste problem (see Section 7 for further information). Furthermore, initial trails done by the processing industry indicated that opening and separation of the meat from the shells was not possible with current processing practises from the blue mussel processing. However, within recent years, studies from other countries of High Pressure Processing (HPP) technology for bivalve mollusk processing have shown interesting results in relation to food safety and quality (e.g., Bonfim et al. 2019).

In the following two chapters, the results of initial trails of using HPP technology for opening non-commercial sized oysters is reported as well as the results from pilot tests of developing new food products based on oyster meat from large and/or clumps of Pacific oysters.

9.1 Using High-Pressure Processing to open clumps and large Pacific oysters

Opening of Pacific oysters using HPP technology

Samples of large Pacific oysters and clumps of oysters often with macroalgae attached (Figure 9.1.1) were collected by hand from shores in Limfjorden in April and August 2019 and subsequently packed and sent cooled alive to HPP Tolling, Ireland, where they arrived two days later.

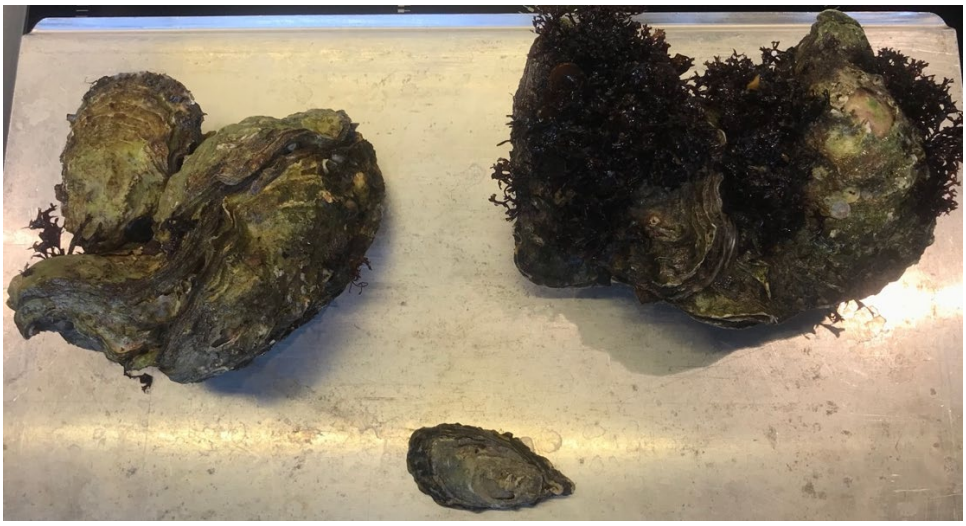

Figure 9.1.1. Two different random selected clumps of Pacific oysters used in the food experiments at DTU SkyLab, FoodLab. The small individual Pacific oyster represent a typical sized oyster served raw at restaurants (Photo: DTU SkyLab FoodLab).

The collected Pacific oysters were divvied into two overall categories of either individual oysters or clumps of oysters, where each category was divided into three subgroups of oysters with shell length <10, 10-15 and >15 cm or clumps of 2, 3-5 or >5 oysters, respectively. All six categories of oysters were tested at six different pressures as 100, 200, 300, 400, 500 and 600 MPa all with a hold time of one minute. The oysters did not open at all at 100 and 200 MPa. At 300 MPa a small number opened. All individual oysters were open at 400 MPa and nearly all clumps of oysters were also open (Table 9.1.1). Furthermore, the pressure causes the oyster meat to turn a bit whiter in colour and plump up a bit (Figure 9.1.2). Further increase in the pressure caused the oyster meat to get very white and plumped up (600 MPa). In conclusion 400 MPa for 1 minute opened most of the oysters with minimal changes to the meat. Generally, the meat is easy to separate from the shells after they have been opened with HPP technology. Furthermore, only 1.2% of the oysters had shell damages, which reduces the risk of presence of shell fragments in the meat.

Table 9.1.1. Numbers of Pacific oysters within each of the six categories that are open, slightly open or closed after exposure to 400 MPa for one minute. The changes in meat quality are also noted.

	400 MPa						
Individuals	**Total number**	**Open**	**Slightly open**	**Closed**	**Size of closed**	**Meat quality**	**Comments**
Small <10 cm	13	13	0	0		Slightly whiter	All open
Medium 10-15 cm	8	8	0	0		Slightly whiter	All open
Large >15 cm	6	6	0	0		Slightly whiter	All open
Oyster clumps							
Small 2 oysters	7	7	0	0		Slightly whiter & plumped up	All open
Medium 3-5 oysters	4	3	0	1	15 cm	Slightly whiter & plumped up	Mostly open
Large >5 oysters	3	2	1	0		Slightly whiter & plumped up	Nearly all open

Figure 9.1.2. Left: The large clumps of Pacific oysters were opened at 400 MPa. Right: Meat from the large clumps of Pacific oysters after the 400 MPa treatment (Photo: HPP Tolling).

Qualitative assessment of the meat quality

A visual comparison of oyster meat taken out of the shells from oysters opened by either freezing alive oysters or opened by HPP (400 MPa). The comparison of the meat after thawing shows that there is a clear visual difference between meat processed with HPP and subsequently frozen compared to meat that has only been frozen. The meat processed with HPP was more brownish, had less

firm meat texture and had a higher water content than frozen meat that had not been HPP processed. It is therefore recommended that the HPP processed oyster meat should be used in products with further processing, e.g., cooking or marinating, as the visual and textural changes of the meat makes it less suitable as unprocessed food (per. comm. R. Flore, DTU FoodLab).

Cost estimations for opening oysters with HPP technology
An estimate of the cost of using HPP technology to open large individuals and clumps of Pacific oysters is approx. DKK 4 per kg of alive oysters, which is only the cost for the HPP processing and does not include costs for fishing, transport, separation of meat from the shells or additional costs for further processing of the meat into final products.

9.2 Pilot test of new food products by utilising large and clumps of Pacific oysters

Authors: Pernille Nielsen (ed), Katla Hrund Björnsdóttir and Roberto Flore

All Pacific oysters were collected by hand in the Limfjorden in March 2019 and send cooled and alive to DTU SkyLab FoodLab, where different processing and product pilot tests were carried out by Katla Hrund Björnsdóttir and Roberto Flore. A detailed report (in Danish) of their studies can be found in Appendix 9.1. The flowing tests were carried out:

- Opening of individual Pacific oysters in the clumps by shucking
- Preparation of frozen Pacific oysters by steam and vacuum
- Marinating whole Pacific oyster meat clumps
- Freeze-drying of whole oyster meat clumps and production of oyster powder
- Dehydration of whole oyster meat clumps and production of oyster powder
- Owen drying of minced oyster meat and production of “oyster snack crackers”.
- Distillation of oyster alcohol

Summary of the results from the DTU SkyLab FoodLab
The shucking of large individuals or clumps of Pacific oysters was time consuming and cumbersome. In addition, shell fragments, which are difficult to remove, often enter the oysters and resulted in an unpleasant feeling when eating the raw oysters. Freezing of the oysters made the shucking of oyster relatively easy afterwards and it did not seem (visual inspection) to affect the texture of the meat, but this has not been investigated further. The processed Pacific oysters contained algae in their digestive system, which gave them an unpleasant appearance and texture when eating them raw. However, the algae gave them a sweet and fresh taste that could be desirable as food additives and/or in distilled beverages. However, further research is needed to optimize recipes and methods before any of the above processing methods or products are ready for commercial utilization/product development.

9.3 Conclusion

Using the HPP technology to open large sized or clumps of Pacific oysters was very successful in terms of opening, had low shell damage but the meat was slightly whiter and plumped up. After freezing and thawing the texture of the meat changed and meat from oysters opened with HPP technology is therefore recommended to be used in processed products. The different processing and product pilot tests of alive large sized and clumps of oysters done by DTU SkyLab FoodLab showed interesting results, but further development of both the tested products and processing methods are needed before the large sized and clumps of oysters can be utilised commercially.

9.4 References

Bonfim RC, de Oliveira FA, de Oliveira Godoy RL, Rosenthal A (2019). A review on high hydrostatic pressure for bivalve mollusk processing: relevant aspects concerning safety and quality. Food Science and Technology. SBCTA; 2019;39: 515–523. doi:10.1590/fst.26918.

10. Recommendations

Pacific oysters in Danish waters have within the last three decades mainly been in the Limfjorden, Isefjord and the Wadden Sea, however Pacific oysters have within the last three to five years also been observed in multiple locations at shallow waters along the coastline of the East coast of Jutland, Zealand and Funen[12]. Studying the development of the existing Pacific oyster populations in the three areas where they have existed for more than 25 years provide useful information for how Pacific oysters potentially would evolve over time in the newly colonised areas, their impact and treats to native species and local ecosystem and what mitigations measures that could be initiated in relation to e.g., preserving existing bivalve fishing grounds, mitigating spread in Natura 2000 sites or areas of public interest e.g., the use of and access to and from the coast for e.g., walks along the beach, bathing, kayaking, anglers and fishing.

Within the present study, new knowledge has been achieved regarding distribution and expansion of Pacific oysters in the Limfjorden and Isefjord surveying both shallow and deep areas, the genetic status and the screening for bivalve pathogens of the population of Pacific oysters in Danish waters (Limfjorden, Isefjord and the Wadden Sea) have been carried out for the first time. To mitigate the effects of Pacific oysters, two new tools (mini-dredge and sorting equipment) have been developed, tested and evaluated. A floating excavator has also been tested and evaluated as a mitigation tool to remove Pacific oysters in shallow areas. Furthermore, the HPP technology was successfully used to open non-commercial sized (large individuals and clumps) Pacific oysters and a pilot study of potential products based on non-commercial sized oysters was also carried out.

Based on the conclusions of the project, the following recommendations related to development of a sustainable Pacific oyster fishery to mitigate the effects of this invasive marine species in the Danish coastal waters have been formulated:

- The earlier during the establishment of Pacific oysters in an area, the less destructive mitigation measures are needed to remove Pacific oysters. The more complex the population structure gets (from smaller individuals at low densities to higher densities and clumps ending with high-density reefs), the more destructive tools are needed, and the Pacific oysters are of less or no commercial value. To be able to initiate mitigation measures early, systematic monitoring efforts are required both in relation to population development (e.g., biomass, recruitment patterns, densities and genetics), but also in relation to spread of bivalve pathogens to determine if the spread takes place in and to other areas with other important commercial bivalve species both in relation to protection of commercial fishery interests and in relation to nature conservation management.

- Site-specific conditions e.g., water depth and population structure both between and within areas with Pacific oysters will determine the type of mitigation tool which can be used and thereby control the environmental impact of the mitigation action. Within the project, three different mitigation tools were tested. The mini-dredge is recommended in shallow areas with low densities of individuals and smaller clumps, whereas the floating excavator are recommended in shallow areas with higher densities and clumps and could potentially also be used in reef areas. The sorting equipment can be used on-board larger vessels to sort mixed catches of blue mussels and Pacific oysters. Whether the sorting equipment can sort mixed

[12] Notat om udbredelse og påvirkning af stillehavsøsters 2021 udarbejdet af DTU Aqua til Miljøstyrelsen 2021.

catches of Pacific oysters and flat oysters has not been tested. The sorting equipment could potentially also be used on land to sort mixed catches from the mini-dredge e.g., at the landing sites.

- Implementation of fisheries in shallow areas (<3 m) will need to comply with the relevant legislations. Licences for fisheries with the mini-dredge in shallow areas will overlap with the current depth limit for eelgrass in Danish coastal areas and the fishery can therefore not be regulated by the general depth limit of 3 m for bivalve fisheries in the Limfjorden[13]. It is therefore recommended that the eelgrass is protected by site-specific "eelgrass boxes", where fishing is not allowed. However, the general buffer zone of 100 m around eelgrass beds (based on a wire length of <100 m in deeper areas) will likely eliminate most areas with Pacific oysters that can be fished with the mini-dredge. During the scientific fishing carried out by DTU Aqua, the wire length was typically <15 m, which indicates that the general buffer zone of 100 m could be reduced. It is therefore recommended that the general buffer zone of 100 m is adjusted to reflect the specific conditions in the shallow areas, where the wire length of the mini-dredge is shorter and Pacific oysters can be located in shallower areas (closer to shore) than the eelgrass beds.

- According to the current licences, discard of invasive species is not allowed and therefore all caught oysters must be landed. The Pacific oysters of non-commercial interest (large individuals and clumps) are therefore often considered a waste problem by the fishers and traders. Development of cost-efficient processing methods of Pacific oysters, new products and new export markets are required to be able to utilise the large individuals and clumps of Pacific oysters but potentially also utilisation of the caught shells. By-catch is especially a challenge for the fishery with the mini-dredge that will need to be carried out by smaller boats (<10 m), which have limited space for sorting of the catches. A better commercial utilisation of the total catch (incl. large individuals, clumps, and shells) would potentially improve the cost-efficiency of the mini-dredge fishery.

- Increasing local awareness in the municipalities where the Pacific oysters can influence recreational activities could potentially be an effective and low-cost mitigation action. Stakeholder initiatives can be initiated by e.g., informing in local medias or with stands at local shellfish festivals, teaching high-school and school classes or encourage local municipalities, environmental NGOs and environmental authorities to the engage local stakeholders operating in the shallow areas (e.g., fishers, anglers, spear fishers and kayak clubs) in coordinated actions to clean selected areas from Pacific oysters.

[13] BEK nr. 2298 af 03/12/2021.

11. Appendices

Appendices are published in a separate report on DTU Aqua's homepage https://www.aqua.dtu.dk/english/about/publications/reports/since-2008

Appendix 2.1: Monitoring and distribution of Pacific oysters in the Limfjorden and Isefjord

Appendix 3.1: Student report: Automatic detection and estimation of submerged oysters from drone images

Appendix 3.2: Student report: Methods to estimate oyster coverage by using different algorithms

Appendix 4.1: Genetic tables

Appendix 7.1: Stakeholder interview questions

Appendix 8.1: Tables mini-dredge assessment

Appendix 8.2: Tables floating escavator assessment

Appendix 9.1: Gastronomic possibilities of large Pacific oysters

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