Gefährdungspotential der eulitoralen Miesmuschelbänke im Niedersächsischen Wattenmeer durch die Bioinvasion der Pazifischen Auster (*Crassostrea gigas*)

Endangering potential of eulittoral Blue mussel beds in the Wadden Sea of Lower Saxony by the bio-invasion of the Pacific oyster (*Crassostrea gigas*)

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Contents

Preface

This dissertation is composed of four publications and one additional chapter as listed below. It further includes a general introduction and a synoptic discussion. It is the main part of a project investigating the bioinvasion of the Pacific oyster (*Crassostrea gigas*) into the East Frisian Wadden Sea, funded by the "Niedersächsische Wattenmeerstiftung" (Project 7/02).

Chapter 1

Schmidt A, Wehrmann A, Dittmann S

Population dynamics of the invasive Pacific oyster *Crassostrea gigas* during the early stages of an outbreak in the Wadden Sea (Germany).

The concept of this study was developed together with the second and the third author. I carried out the investigations and evaluated the results. The manuscript was written by me with editorial advice by Dr. S. Dittmann. This article was published 2008 in Helgoland Marine Research 62:367–376

Chapter 2

Schmidt A, Herlyn M, Millat G, Wehrmann A, Dittmann S

Comparison of the population dynamics of the invasive Pacific oyster (*Crassostrea gigas*) with the native Blue mussel (*Mytilus edulis*) in the Wadden Sea (Germany).

The idea of this study was devised by me. The second and third authors supplied the data of the Blue mussels. I performed the analysis, and both evaluated the results and wrote the manuscript with editorial advice by Dr. S. Dittmann. This article was submitted to Biological Invasion

Chapter 3

Schmidt A, May P, Wehrmann A, Dittmann S

Spatial overlap and feeding competition between an introduced and an indigenous epibenthic bivalve on tidal flats in the southern North Sea

The investigations for the spatial overlap were carried out by me. The experiments for the feeding competition analysis were part of a diploma thesis of P. May who was supervised by me. The manuscript was written by me with scientific and editorial advice by Dr. S. Dittmann. This article is prepared for submission.

Chapter 4.1

Pradillon F, Schmidt A, Peplies J, Dubilier N

Species identification of marine invertebrate early stages by whole-larvae in situ hybridisation of 18S ribosomal RNA.

The described method for species identification was developed together with the first author, whereas the method with use of the DIG labelled probes was developed by me. The third author did preliminary examinations and together with the fourth author scientific an editorial advice. I was also involved in the writing of the manuscript. This article was published 2007 in Marine Ecology Progress Series 333:103-116.

Chapter 4.2

Schmidt A

Pacific oyster identification by whole-larvae in situ hybridisation – improved method of (Pradillon et al. 2007) – for the direct use in a plankton sample

The idea and the development of the improved method were carried out by me and I wrote the manuscript.

Summary

In a world of increasing globalisation nearly all ecosystems become threatened or are already affected by non-indigenous species, which were translocated through human activities. When a non-indigenous species reaches a new region, it is not predestined if it will become invasive or a pest for the region. But if an invasive species is successful, this can lead to massive alterations in the recipient region. The best examples are the bio-invasions which occurred in New Zealand or Australia, e.g. the introduction of the rabbit to Australia. In the marine area, for example, the Mediterranean Sea was harmed by many non-indigenous species one of the worst examples is the algae *Caulerpa taxifolia*. How species are translocated is well known, but how they adapt into a new region and what impact these new species have to the ecosystem needs further investigations, especially because of more and more new occurring bio-invasions. In most cases a non-indigenous species and the impact it has is recognised after it is too late to avert the bio-invasion. Therefore, attention should be paid to the issues how invasive species could be discovered and how a bio-invasion proceeds within a recipient region.

The Pacific oyster (*Crassostrea gigas* Thunberg 1793) presented an ideal research object to investigated bio-invasion. The oyster shows a worldwide distribution and many investigations were already done, therefore it functions as a good model organism and can deliver a comprehensive picture of bio-invasions in the marine environment. In the course of the worldwide transport of the Pacific oyster, mainly for aquaculture purpose, the oyster was also deliberately introduced for aquaculture to the southern North Sea (in the Oosterschelde, The Netherlands) in 1965. Environmental factors, such as optimal temperature for growth and reproduction and water currents, allowed the spread of the Pacific oyster into the Dutch Wadden Sea in 1983. Later, since the late 1990's, the Pacific oyster has spread further into the East Frisian Wadden Sea (Germany).

One of the main issues of this study was to investigate the population dynamics of an early invasive spread of a non-indigenous species within a new region. It was possible to investigate this because of early single findings of Pacific oyster specimens in the East Frisian Wadden Sea. The study was conducted between the years 2003 and 2005. Investigations

were done on Blue mussel beds (*Mytilus edulis* beds), because first specimens of the oyster, a hard substrate inhabitant, were found on Blue mussel beds, which provide the predominant secondary hard substrate in the tidal flat environment of the Wadden Sea. In the first study year a west to east gradient of high abundances in the west and low abundances in the east indicated an eastward directed spread of the oyster, coming from the Netherlands. In the second and third year abundances of the Pacific oyster increased and were more heterogenic and differed between adjacent tidal basins. The population increase of the Pacific oyster reached levels similar to native occurring bivalve populations, with a growth constant (*K*) that varied between 0.300 y^{-1} and 0.990 y^{-1}. At the same time mortality (*Z*) for young cohorts (between half and one and a half years old) was low (*Z* = 0.03 y^{-1} to 0.13 y^{-1}). The highest Pacific oyster density found on a mussel bed had a mean abundance of > 300 ind. m^{-2}, and a maximum of 1460 ind. m^{-2}. Additional investigations showed that the ongoing spread of the Pacific oyster is enhanced by their preference to settle on the shells of conspecifics. Also the water temperature, the main factor influencing the propagation of the oyster (the assumption that the water temperature was too low in the North Sea was the reason to allow the Pacific oyster cultivation), was measured. In the three consecutive years of this study the water temperature in summer was above 19.5 °C and therefore high enough for oyster development.

For further investigations on how species spread within a region, a molecular method was developed for an easy species identification of marine invertebrate early stages. In case of the Pacific oyster this method should be used to identify dispersal patterns of oyster larvae within the Wadden Sea.

Further main issues of this study were to investigate the influence of the non-indigenous Pacific oyster on the native Blue mussel and assess the influence on the Wadden Sea ecosystem. At first, the high density of the oyster suggested a displacement of the native Blue mussel. However, a comparison of the population dynamics of oyster and Blue mussel showed no negative effect of the establishment of the Pacific oyster population on the native Blue mussel population. If there is no negative effect on population level, it might be possible that the two species compete for space on a small scale. The spatial overlap was estimated

by analysing the density of oysters and mussels. The data showed that Pacific oysters settled in areas which were formerly occupied by Blue mussels. However, a positive correlation of oyster and mussel densities indicated a coexistence of both species. Furthermore, this co-occurrence can lead to food competition in densely populated oyster / mussel beds. To estimate the competition for food, the filtration rates of both species were analysed, and showed a higher filtration rate for Blue mussels than for Pacific oysters, indicating an advantage in filtration efficiency for the mussel. This filtration benefit of the Blue mussel explains the ability of the smaller mussel to live deep inside the structure of the larger oysters, which was the case on many mixed mussel beds. To live inside the structure of the oysters can give shelter to Blue mussels (probably as well as to other species) and therefore protection against e.g. predators, due to their complex structure. One important example, which could influence the sustainability of the ecosystem, is the potential loss of the mussel beds as food source for birds foraging on mussel beds.

At present, Pacific oysters are well established at several locations in the East Frisian Wadden Sea. Considering the present growth of the Pacific oyster population, it is most likely that the oyster population in the Wadden Sea becomes self-sustaining and, furthermore, all former mussel beds of the tidal flats will be replaced by mixed mussel / oyster beds in the future.

Zusammenfassung

Durch eine weltweit zunehmende Globalisierung sind heute nahezu alle Ökosysteme durch fremde, von dem Menschen verbreitete, nicht einheimische Arten bedroht. Es ist allerdings nicht gesagt, ob sich eine eingeführte Art ausbreitet oder sogar zu einer Plage wird. Wenn aber eine nicht heimische Art es schafft, sich in einem neuem Gebiet auszubreiten, kann dies massive Veränderungen zur Folge haben. Mit die bekanntesten Beispiele von Bio-Invasionen gibt es in Neuseeland oder Australien, wie zum Beispiel die Invasion der Kaninchen in Australien. Im marinen Bereich ist das Mittelmeer ein Gebiet, welches stark durch nicht heimische Arten beeinflusst ist, zu den berühmtesten Beispielen gehört mit Sicherheit die Alge *Caulerpa taxifolia*. Auf welchen Wegen Arten durch den Menschen in neue Gebiete gelangen, ist mittlerweile gut bekannt - wie sie sich an ihre jeweilige neue Umgebung anpassen und welchen Einfluss sie dort haben, ist allerdings noch nicht in allen Einzelheiten untersucht. Meistens werden neue Arten und ihr Einfluss erst bemerkt, wenn es zu spät ist um die Ausbreitung noch zu verhindern. In Folge dessen sollte mehr Augenmerk auf die Untersuchung von Bioinvasionen, wie man sie entdecken und untersuchen kann, gelegt werden, sowie auf die Frage wie Bio-Invasionen von Statten gehen.

Für die Untersuchung von Bio-Invasionen stellt die Pazifische Auster (*Crassostrea gigas*, Thunberg 1793) ein ideales Forschungsobjekt dar. Vor allem, da die Auster bereits weltweit verbreitet ist und an ihr schon viele Untersuchungen durchgeführt wurden, ist sie ein idealer Modellorganismus der ein umfassendes Bild über Bio-Invasionen im marinen Bereich liefern kann. Die Auster wurde weltweit vorwiegend durch Aquakultur verbreitet, und fand hierdurch auch ihren Weg in die Nordsee, wo sie 1965 in Holland für die Aquakultur in den Oosterschelden eingeführt wurde. Von da aus, breitete sich die Auster Richtung Osten aus und ist seit den späten 1990er auch im Ostfriesischen Wattenmeer (Deutschland) zu finden.

Eines der Hauptziele dieser Arbeit ist es, die Populationsdynamik einer nicht einheimischen Art zu untersuchen, die im Beginn ist sich in einem neuem Gebiet auszubreiten. Solch eine Untersuchung wurde ermöglicht durch die frühzeitigen Funde einzelner Austern im Ostfriesischen Wattenmeer. Die Untersuchungen fanden im Zeitraum von 2003 bis 2005 statt. Da die Auster, ein Hartsubstrat-Bewohner, zuerst auf

Miesmuschelbänken (*Mytilus edulis* Bänken) gefunden wurde, welche auch das vorwiegende sekundäre Hartsubstrat im Wattenmeer zur Verfügung stellen, wurden die Untersuchungen dort durchgeführt. Im ersten Untersuchungsjahr wurde ein Gradient der Austernabundanz von West nach Ost gefunden, mit einer hohen Abundanz im Westen und einer niedrigen Abundanz im Osten. Dies weist auf eine aus Holland kommende, ostwärts gerichtete Ausbreitung der Auster hin. Im zweiten und dritten Untersuchungsjahr nahm die Abundanz der Auster zu, zeigte aber eine eher heterogene Verteilung mit unterschiedlich hohen Abundanzen zwischen benachbarten Gezeitenbecken. Das Populationswachstum der Auster war ähnlich hoch wie das von natürlichen Populationen, die Wachstumskonstante (*K*) lag dabei im Bereich von 0,300 Jahr^{-1} bis 0,990 Jahr^{-1}. Die Mortalität (*Z*) dagegen war niedrig (*Z* = 0,03 Jahr^{-1} bis 0,13 Jahr^{-1}) für junge, ein halb bis eineinhalb Jahre alte Kohorten. Die größte Anzahl an Pazifischen Austern, die in den drei Jahren auf einer Miesmuschelbank gefunden wurde, hatte eine mittlere Abundanz von > 300 ind. m^{-2} und ein Maximum von1460 ind. m^{-2}. Weitere Untersuchungen zeigten, dass die weitere Ausbreitung der Auster unterstützt wird durch ihre Vorliebe, sich auf Schalen der eigenen Art anzusiedeln. Des Weiteren wurde die Wassertemperatur auf den Muschelbänken bestimmt, da die Wassertemperatur einer der wichtigsten Faktoren ist, der die Vermehrung der Auster beeinflusst. Wegen der Annahme, dass die Auster sich aufgrund der angeblich zu niedrigen Temperatur in der Nordsee nicht vermehren kann, wurde ihre Kultivierung erlaubt. In den drei Untersuchungsjahren erreichte die Wassertemperatur im Sommer immer Werte über 19,5 °C; welches die Temperatur ist, die die Auster benötigt, um sich zu vermehren.

Für weitere Untersuchungen im bezug auf die Ausbreitung von Arten innerhalb eines Gebietes wurde eine molekular biologische Methode entwickelt für eine einfache Bestimmung von Jungstadien mariner Invertebraten. Im Fall der Austern Bioinvasion sollte die Methode verwendet werden, um die Ausbreitungsmuster der Austernlarven im Wattenmeer zu untersuchen.

Ein weiteres Hauptziel dieser Arbeit war es, den Einfluss der nicht heimischen Pazifischen Auster auf die heimische Miesmuschel zu untersuchen und ihren Einfluss auf das Ökosystem Wattenmeer zu bestimmen. Durch die große Anzahl der Auster wurde zu Beginn

der Invasion vermutet, dass sie die Miesmuschel verdrängen könnte. Der Vergleich der Populationsdynamik beider Arten zeigte jedoch, dass die Austernpopulation keinen Einfluss auf die Miesmuschelpopulation hatte. Auch wenn kein negativer Effekt auf Populationsniveau festzustellen war, könnte es dennoch zu einer Raumkonkurrenz kommen. Die räumliche Überlappung wurde deshalb untersucht, hierfür wurde die Dichte beider Arten auf einer Muschelbank untersucht. Die Daten zeigten dabei, dass die Auster sich in den Bereichen angesiedelt hat, in denen vorher hauptsächlich die Miesmuschel zu finden war. Eine positive Korrelation der Austern- und Miesmuscheldichte deutet jedoch auf eine Koexistenz der beiden Arten hin. Diese Koexistenz könnte allerdings auf einer dicht besiedelten Muschelbank zu einer Nahrungskonkurrenz führen. Um eine mögliche Nahrungskonkurrenz feststellen zu können, wurden die Filtrationsraten der Pazifischen Auster und die der Miesmuschel bestimmt. Dabei zeigte die Miesmuschel eine höhere Filtrationsrate als die Auster, was auf einen Vorteil der Miesmuschel in der Filtrationseffizienz hinweist. Dieser Filtrationsvorteil der Miesmuschel erklärt, dass sie sich als kleinere Art, in den Zwischenräumen der Struktur die durch die Auster entsteht, ansiedeln kann. Dies wurde auf vielen gemischten Muschelbänken (Auster / Miesmuschel) gefunden. Dadurch dass sich die Miesmuschel zwischen den Austern ansiedelt, kann sie, bedingt durch die Struktur einer Austernbank, dort Schutz finden, z. B. vor Räubern. Ein Beispiel, dass die Auster einen Einfluss auf das Ökosystem Wattenmeer haben könnte, ist der Verlust der Miesmuschelbänke als Nahrungsquelle für Vögel.

Derzeit ist die Pazifische Auster in vielen Gebieten des Ostfriesischen Wattenmeeres verbreitet und etabliert. Bedenkt man das derzeitige Wachstum der Austernpopulation, so ist es möglich, dass die Auster eine Populationsgröße erreicht, die sich selbst erhalten kann und alle früheren Miesmuschelbänke zu gemischten Austern / Miesmuschelbänken werden.

General Introduction

Bio-invasion has become an increasing problem in ecosystems of the world. Particularly the 'globalisation' with intercontinental shipping facilitates transport of organisms, e.g. in ballast water or as attachment to ships' hulls, and increased the number of non-indigenous species (Carlton 1985, Carlton & Geller 1993, Minchin & Gollasch 2002, Ruiz et al. 2000). Canals create new connections between oceans (Lodge 1993). Furthermore, species introductions, accidentally or intentionally, occur via aquaculture, including species that were brought in unintentionally as stowaways such as epifauna and -flora as well as parasites and pathogens (Carlton 1996c, Chew 1990, Lodge 1993, Naylor et al. 2001, Wolff & Reise 2002). Invasive species can have a lasting impact on biodiversity and ecosystem function (Carlton 1996c, Carlton & Geller 1993, Crooks & Khim 1999, Mack et al. 2000, Ruiz et al. 1999). The impact of an invasive species on a new region can be substantial, but to become invasive the species must be successful in the recipient region. One factor supporting invasions worldwide, which has been frequently discussed, is climate change (Stachowicz et al. 2002).

In any case, bio-invasions generate many issues dealing chiefly with the way of introduction and possible impacts of the invaders. Research, for instance, was done to identify the attributes of species that predispose them to become invasive (e.g. Carlton 1996c, Lodge 1993), to know how species can reach or disperse into new regions (e.g. Carlton 2003, Gollasch et al. 2003), and to determine the impact of an invader in the recipient ecosystem (e.g. Carlton 1996b, Grosholz 2002). Species with r-selected life history characteristics (rapid growth, early maturity, short life spans, high fecundity, and extensive dispersal capacity) are especially successful invaders because of their ability to build up high abundances shortly after introduction (Lodge 1993, McMahon 2002, Williamson & Fitter 1996b). Therefore, investigating the characteristics of invaders and recipient communities is important (Carlton 1996c, Crawley 1987, Di Castri 1990, Kolar & Lodge 2002, Lodge 1993, McMahon 2002, Ruiz et al. 1997), especially to develop prediction models about bio-invasion (Branch & Steffani 2004). Hence, it is important to learn from previous invasions to identify and predict new invaders and their impact. In particular, if a species was established and has spread from its point of introduction, eradication is almost impossible (Mack et al. 2000) and leads to many

direct and indirect effects on the native community, ranging from species level consequences to impacts on food-web properties and ecosystem processes as shown by numerous examples (see review of Grosholz 2002).

The knowledge about marine bio-invasions in recipient communities is not comprehensive and most studies describe either the current state (e.g. Grizel and Héral 1991; Mann and Harding 2000; Streftaris et al. 2005) or a change over several years (e.g. Diederich et al. 2005; Escapa et al. 2004; Herkül et al. 2006; Oliveira et al. 2006), whereas there is little evidence about the patterns of spread during the early phase after a species became invasive and starts to spread. Therefore, this case study about the invasion of the Pacific oyster *Crassostrea gigas* in the southern North Sea intends to improve the knowledge about population dynamics in the early phase of a bio-invasion. In particular, the Pacific oyster was rare or nonexistent in most parts of the study area during preliminary surveys in the western part of the East Frisian Wadden Sea (Wehrmann et al. 2000). As it was known from other areas in the Wadden Sea, e.g. in The Netherlands, that the oyster was a successful invader (Dankers et al. 2004), a further spread into the East Frisian Wadden Sea was expected. Furthermore, the Pacific oyster is an excellent model organism for marine bio-invasion studies as it is well known as introduced and successful species throughout the world which is supported by aquacultures and its ability to adapt to a wide range of environmental situations (Chew 1990).

Prior to the introduction of the Pacific oyster, the North Sea coast has already been affected by about 80 non-native species that became established (Reise et al. 2002). Although some of these species may have influenced the ecosystem, most remained insignificant additions to the native biota (Reise et al. 2005). The latter was also predicted for the Pacific oyster based on the argument that this species would not be able to reproduce because of its natural distribution in relatively warm waters, whereupon the oyster was intentionally introduced for aquaculture (Drinkwaard 1999). The introduction of the Pacific oyster intended to substitute the European oyster *Ostrea edulis*, which became extinct in the southern North Sea due to overexploitation, disease and cold winter (Reise 1998).

Figure 1: Blue mussel bed (top) and Pacific oyster reef (bottom) in the East Friesian Wadden Sea in 2003.

However, unlike previous bivalve invasions by the soft shelled clam *Mya arenaria* and the Razor clam *Ensis americanus*, the Pacific oyster is more likely to change the habitat structure in the Wadden Sea (Figure 1), affecting blue mussel beds (*Mytilus edulis*) and their associated organisms. The Pacific oyster functions as an ecosystem engineer with the ability to alter habitat characteristics by forming massive epibenthic reefs and, furthermore, its introduction may have community-level effects proportional to its abundance (see Jones et al. 1994, Reusch & Williams 1999). So far, several studies were carried out to investigate the bio-invasion of the Pacific oyster in the North Sea (e.g. Dankers et al. 2004, Diederich et al. 2005, Reise 1998), but the early phase of this bio-invasion remained unclear and is in general a knowledge gap in the field of marine bio-invasions. Therefore, this study was conducted to investigate the population dynamics and the impact of an invasive species during the first years of a bio-invasion exemplary on the invasion of the Pacific oyster into the East Friesian Wadden Sea.

Human mediated marine invasions

Marine invasions caused by humans happened for at least several thousand years (Carlton 1999a), when man started to move across natural barriers. The first known introduction of a marine species was done by the Vikings around 1250, who introduced the soft-shelled clam to Europe (Petersen et al. 1992). The distribution of marine species has been altered dramatically since at least the 14th century, and definitely since the journey of Columbus in 1492, which marks the beginning of the modern seafaring. Species introductions after the journey of Columbus are described as ‘neozoen’, and species introductions before Columbus as ‘archaeocoen’. A further change in species translocation occurred through the ongoing globalisation, which lead to an increase of species that were transported around the world. For illustration, while in the period between 1500 and 1800 only approximately three species a year were spread, now nearly 1000 coastal species of marine organisms are regarded as cosmopolitan (Carlton 1999b).

Vectors, by which species can move through human activities, include vessels (e.g. hull fouling, solid ballast, ballast water), fisheries and mariculture (e.g. deliberate and / or

accidental with translocation), aquarium trade, intentional or accidental releases into the wild, man-made canals (natural range expansion), deliberate and / or accidental with plant translocation, as well as deliberate and / or accidental with translocation for biocontrol and science (release as result of research activities) (Carlton 1999a, Hewitt & Hayes 2002). In each case, the vector transports species from a donor region into a recipient region, where specimens are released. Depending on the region, specific geographic patterns determine the predominant origin of most invasive species. For instance, from 38 molluscs introduced in the Northern Hemisphere 63 % originate in the North Atlantic Ocean / Mediterranean area, whereas 37 % originate in the North Pacific Ocean. Within the Atlantic Ocean, the North-western Atlantic is a significantly stronger donor area, providing 75 % of those taxa which dispersed globally. For the Pacific Ocean the western Pacific is also the predominant donor region, exporting 93 % of all those species originating in the Pacific (Carlton 1999a).

Beside the neozoon and archaeozoon, cryptogenic species are not demonstrably native or introduced (Carlton 1996a). Among the species that were introduced to the North Sea coast the soft shelled clam provides an example for an archaeozoon, the slipper shell *Crepidula fornicata* for a neozoon and an example for a cryptogenic species is the macroalga *Fucus evanescens* (Reise et al. 1999).

Fortunately, from the large amount of species moved around the world daily and introduced in regions outside their native range, only few can survive in their new habitat and even less are able to propagate and build up an immense population (Lodge 1993, Williamson & Fitter 1996a, Williamson & Fitter 1996b).

Pacific oyster ecology

Oyster taxonomy

Taxonomic name: *Crassostrea gigas* (Thunberg 1793)

Phylum: Mollusca

Class: Bivalvia

Subclass: Pteriomorphia

Order: Ostreoida

Suborder: Ostreina

Superfamily: Ostreoidea

Family: Ostreidae

Genus: *Crassostrea*

Common names: giant oyster, giant Pacific oyster, immigrant oyster, Japanese Oyster, Miyagi oyster, Pacific oyster

Figure 2: Pacific oyster juveniles and adults in a cluster.

Shell description

The shell of the Pacific oyster is inequivalve, inequilateral and extremely variable in shape depending on the substrate: on hard substrates the shape is roundish, on soft substrates it has an ovate smooth shell, and on mini-reefs the shell is solid with irregular margins (CIESM 2000). The anterior margin is longer than the posterior (CIESM 2000). The shells are sculpted with large, irregular, rounded and radial folds with overlapping, concentric lamellae in mature specimens (Nehring 2006). The upper (right) valve is flattened with a low round umbo. The lower (left) valve is larger, more convex having a well developed umbo (CIESM 2000). The shell colour is usually whitish with purple streaks and spots radiating away from the umbo (Nehring 2006). The common size covers the range of 80-300 mm in length, whereas exceptional specimens can attain 400 mm (CIESM 2000). The largest specimen found in the European Wadden Sea had a length of 310 mm (Reise 2005).

Reproduction and life cycle

The Pacific oyster is an oviparous species with a high fecundity; females produce 20-100 million eggs (diameter 50-60 µm) which are released over several spawning bursts. Spawning occurs at water temperatures of 18.5-24 °C and salinities of 23-28 ‰ (ppt) (CIESM 2000, ISSG 2005, Nehring 2006, NIMPIS 2002). The optimum salinity for the growth of oysters is 25 - 35 ‰ (Quayle 1969). Temperature appears to be the main limiting factor for reproduction in the wild. The oysters are protandrous hermaphrodites, of male gender first and turning into a female after a year. Pacific oysters reach their first reproductive period in the summer one year after settlement (CIESM 2000, ISSG 2005, Nehring 2006, NIMPIS 2002). In northern waters, this happens in July and August (Reise 1998). During the breeding season the reproductive organs may constitute 50 % of the body's volume. Fertilisation is external, takes place in the seawater column, and must occur within 10-15 hours after spawning. Larvae (larval development: see Figure 3) are planktonic and free swimming. The larval period lasts 3 to 4 weeks, dependent of the water temperature. The larvae develop organs that allow them to swim, although water currents remain the dominant means for natural dispersion (CIESM 2000, ISSG 2005, Nehring 2006, NIMPIS 2002). The spread can also be influenced by the

required food for developing larvae and the presence of predators, especially of shore crabs (Eno et al. 1997).

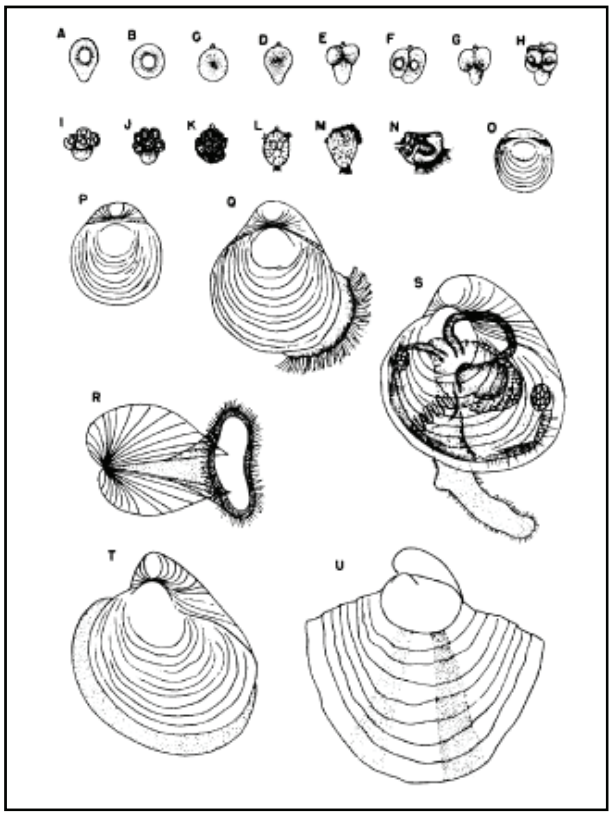

Figure 3: Larvae development of the Pacific oyster. A: Ovarien egg; B:Unfertilized egg; C: Fertilized egg, first polar body discharged; D: First polar lobe appeared; E: First cleavage; F: First polar body resorbed; G: Second polar body appeared; H: Second cleavage; I: Second polar lobe resorbed; J: Third cleavage; K: Morula stage; L: Gastrula stage; M: Trochophore; N-R: Veliger; S: Pediveliger; T: Spat one day after fixation; U: Spat eight days after fixation. (Arakawa 1990)

When settling, the larvae group together and crawl around the sea floor, searching for a suitable hard substratum to which they can cement their left shell valves. The oyster grows on average 25 mm per year. They are able to grow in temperatures from 4 to 35 °C and survive temperatures as low as -5 °C. Mortality is recorded starting at 30 °C, 40 °C for 1 hour results in 100 % mortality. Pacific oysters can live up to 30 years. The Pacific oyster is a filter feeder and ingests bacteria, protozoa, a wide variety of diatoms, larval forms of other invertebrate animals, and detritus (CIESM 2000, Dankers et al. 2004, Nehring 2006, NIMPIS 2002, Quayle 1988).

Habitat

The Pacific oyster occurs naturally in estuarine and coastal marine waters of Japan and south-east Asia. The epifaunal living oyster will attach to almost any hard surface in sheltered waters. Whilst they usually attach to rocks in their native range, the oysters can also be found in muddy or sandy areas. Oysters will settle on adult specimens of the same or other bivalve species, which leads to the development of reef structures (Figure 4). In its native range they prefer sheltered waters in estuaries where they are found in the intertidal and shallow subtidal zones down to a depth of about three meters (CIESM 2000, NIMPIS 2002).

Figure 4: Pacific oyster reef in the East Friesian Wadden Sea (Germany).

Pacific oyster invasion history

The natural habitat of the Pacific oyster is the sea around Japan and Korea. In Japan several strains are known from the Pacific oyster, which are differentiated and characterised by the region where they occur and by factors such as growth, shape of the shell or colour (Quayle 1969). In the north of Japan the Hokkaido type occurs, which is characterised by a vast growth and a gray-white colour. Further south the Miyagi type can be found, which is in colour between the Hokkaido and the more southerly Hiroshima type, characterised by a slower growth and a blackish purple and brown colour. In the far south of Japan a stunted form of the Pacific oyster occurs, the Kumamoto type which is named after the main production area (Quayle 1969).

From Japan, Portuguese explorers transported the Pacific oyster to southern Europe by the 1500s (Carlton 1999a). With beginning of the 20th century the Pacific oyster became transported around the world for aquaculture purpose (see Figure 5), at first in 1902 and further from the 1920s on from Japan to the north west of the USA and to British Columbia, Canada (Chew 1990, Wolff & Reise 2002). From the west coast of North America the Pacific oyster was introduced to Europe in the 1960s and after 1980 to the east coast of the USA, to South America and South Africa. Besides the introduction from North America the Pacific oyster was also imported to Europe from Japan (Chew 1990, Drinkwaard 1999, Grizel & Héral 1991, Wolff & Reise 2002). From the west coast of South America the Pacific oyster was illegally transported to the east coast of Argentina in 1982 (Orensanz et al. 2002). Furthermore, the Pacific oyster was introduced to Australia from Japan in the 1950s, from where the oyster was further introduced to New Zealand (Chew 1990).

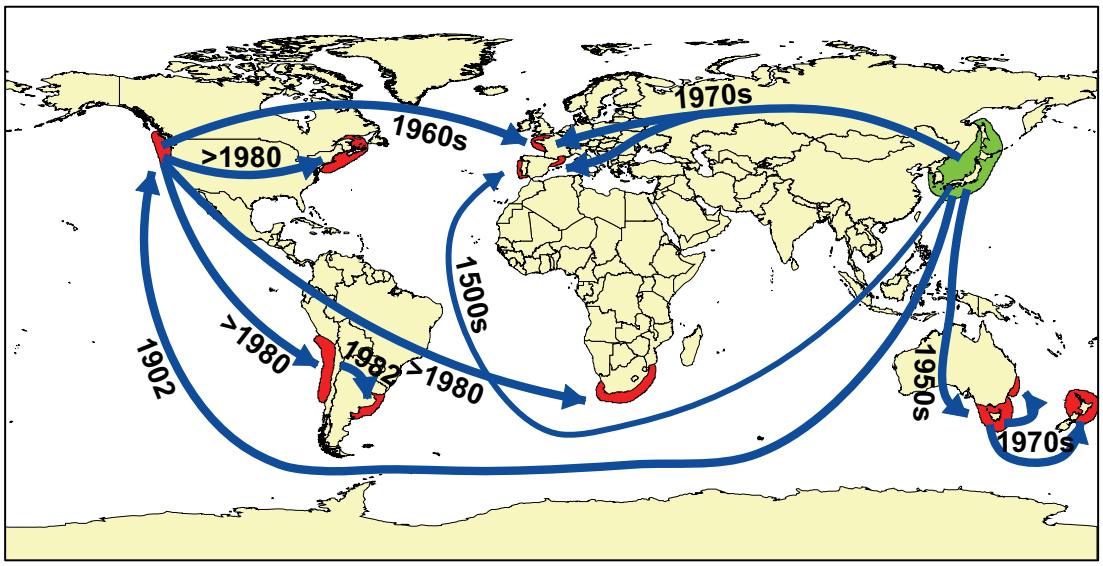

Figure 5: Distribution and years of first introduction worldwide of the Pacific oyster. Green highlighted is the native range and red the areas of introductions. Map after Chew (1990) and Wolff & Reise (2002).

In Europe the Pacific oyster was introduced for aquaculture purposes into several European coastal waters: to Portugal (Chew 1990), France (Grizel & Héral 1991), Great Britain (Walne & Helm 1979), The Netherlands (Drinkwaard 1999) and to Germany (Reise 1998). An overview of introduced non-native oysters is published in Ruesink et al. (2005). The global distribution, including coastal areas where the Pacific oyster is native and where it was introduced, is shown in Figure 6.

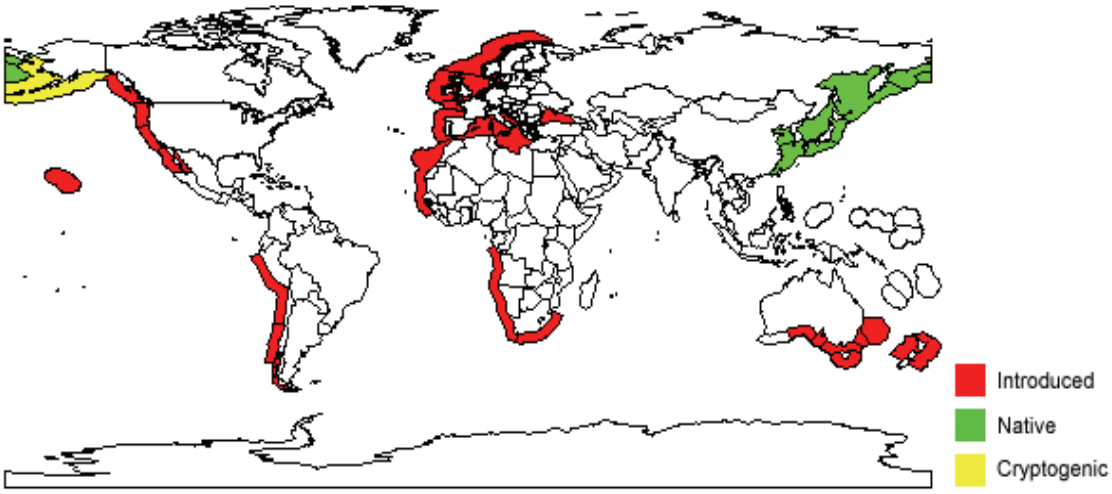

Figure 6: Global distribution of the Pacific oyster (NIMPIS 2002)

Worldwide, in regions where the Pacific oyster was intentionally (e.g. aquaculture) or unintentionally introduced, spatfalls occurred and consequently wild oyster populations arose with a further dispersal by natural means (Andrews 1980, Chew 1990, Ruesink et al. 2005), e.g. North America (Andrews 1979, Quayle 1969), South America (Escapa et al. 2004), South Africa (Robinson et al. 2005), Australia (Ayres 1991, Dix 1991) and Europe: France (Grizel & Héral 1991), The Netherlands (Drinkwaard 1999), Germany (Reise 1998, Wehrmann et al. 2000) and England (Eno et al. 1997).

Before the Pacific oyster was successfully introduced to European coasts, several attempts were made with American oysters *Crassostrea virginica* and Portuguese oysters *Crassostrea angulata* to compensate the over-exploited stocks of the European oyster *Ostrea edulis*, however, these attempts failed (Wolff & Reise 2002). In the southern North Sea the European oyster is extinct due to over fishing, disease and cold winters. Introduction attempts with the Portuguese oyster at the German coast failed as well. Portuguese oysters imported from Portugal and Spain were introduced into the Wadden Sea near Norddeich and into the Jadebusen in 1913-14 and near Sylt in 1954, 1961 and 1964 (Meyer-Waarden 1964, Neudecker 1992, Wehrmann et al. 2000). The Pacific oyster was imported from Scottish hatcheries for aquaculture experiments to different areas in the Wadden Sea (dates and places of Pacific oyster aquaculture attempts: 1974 Neuharlingersiel; 1976/1982 Jade, 1982 Wangerooge, 1987 Norderney (Neudecker 1985, Wehrmann et al. 2000)) and to the German Baltic Sea coast in the Flensburg Fjord (Meixner & Gerdener 1976, Seaman 1985).

The first successful introduction of the Pacific oyster into the southern North Sea (see Figure 7) for aquaculture occurred in the Oosterschelde (The Netherlands) in 1965 (Drinkwaard 1999, Reise 1998). The imported spat of the oysters came from British Columbia and in the following years also from Japan (Nehring 2006, Wolff & Reise 2002). Natural spat falls of the Pacific oyster outside the aquaculture plots occurred for the first time during two exceptionally warm summers in 1975 and 1976 (Drinkwaard 1999, Kater & Baars 2003). A second successful Pacific oyster introduction for aquaculture occurred on the German coast at the island of Sylt, from where a spread throughout the northern German and Danish Wadden Sea started five years after the introduction in 1986 (Diederich 2005, Nehls et al. 2006, Reise

1998). The spat for the aquaculture was primarily taken from British and Irish hatcheries (Nehring 1999, Reise 1998).

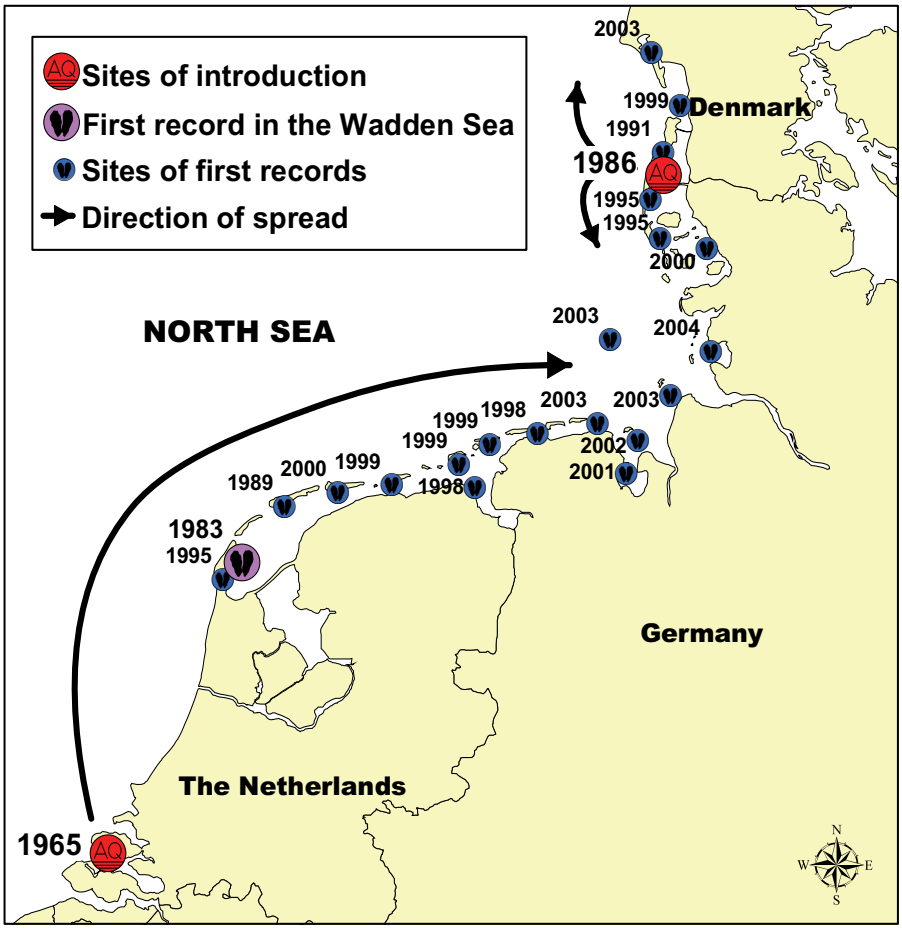

Figure 7: Pacific oyster sites of introduction for aquaculture (AQ) and records of natural spread in the southern North Sea. Map modified from Reise et al. (2005).

After the first recruitment events in the Oosterschelde wild populations originated and abundances increased along the southern North Sea coast. In 1983, Bruins (1983) reported the first Pacific oyster findings in the Wadden Sea attached to stones at Texel, and in 1998 Tydeman (1999) discovered Pacific oysters in the Harbour of Eemshaven (at the Dutch-German border). On the East Frisian coast, the first individuals were found in 1998 (Wehrmann et al. 2000). One theory about the dispersion of the Pacific oyster into the Wadden Sea was described by Wehrmann et al. (2000) as a natural spread of the planktonic larvae flowing eastward with the residual current on the southern North Sea coast, resulting from tidal currents and prevailing winds. Others described that the Pacific oyster was probably

deliberately or accidentally introduced with mussel transports from the Oosterschelde to the island of Texel (Bruins 1983, Nehring 2006, Wolff 2005). In fact, after the oyster arrived at Texel it was frequently observed along the entire Dutch Wadden Sea (Dankers et al. 2004) and started to spread into the East Frisian Wadden Sea.

New method for marine ecology studies – investigation of the dispersal of the Pacific oyster

Molecular ecology had its genesis in the mid-1960s, when protein electrophoresis was first used to detect genetic variation in samples of individuals from different populations and species (see Baker 2000). Not much later attention in molecular biology switched from the translated protein to the DNA level. The use of methods on DNA level had their breakthrough and became mass applicable with the technique of the polymerase chain reaction (PCR). Nowadays, molecular methods are used to study ecological phenomena, from molecular sexing of individuals and parentage of offspring to population structure of species and phylogenetic relationship of taxa. For many studies dealing with population dynamic issues the identification of species is essential, but this is often difficult on the basis of morphology, especially the identification of marine invertebrate and fish larvae with their different stages and similarity to other species (Levin 1990). Therefore, the use of molecular techniques can be an alternative way to identify species. Many techniques for species identification exist and are already in use (for review see Garland & Zimmer 2002), however, most of them are costly, destructive, and not quantitative. For comprehension of bio-invasion patterns of the Pacific oyster, it is necessary to investigate the occurrence of oyster larvae in the plankton to gain knowledge about migration paths (through the tidal flats or offshore in front of the islands), spawning spots, drift rate and larvae input (import of larvae from The Netherlands). A very extensive sampling is essential, which would be very costly when using classic morphology methods. Therefore, a molecular method should be developed for an easy and quick processing of plankton samples, which is not destructive and quantitative.

Objectives of the study

This study has been carried out to gain knowledge about the early phase of a bio-invasion on the example of the invasion of the Pacific oyster into the East Friesian Wadden Sea. The aims were to investigate

(i) the initial spread of a non indigenous species into a new region,

(ii) the population dynamics during the initial spread,

(iii) the influence on the population of a native species which occupies a similar ecological niche,

(iv) and the impact of a non indigenous species on the recipient ecosystem.

Therefore, the population of the Pacific oyster was monitored during the years 2003 – 2005, with additional field surveys for the analysis of oyster growth, settlement and the abiotic environmental conditions, and further laboratory experiments were conducted to analyse the competition for food. A genetic method was developed for an easy identification of the planktonic larvae of the Pacific oyster.

- Chapter 1 deals with the population dynamics of the Pacific oyster
- Chapter 2 compares the Pacific oyster population with the native Blue mussel population
- In Chapter 3 the competition for space and food between the Pacific oyster and the Blue mussel was studied
- In Chapter 4 a genetic method was developed to investigate Pacific oyster larvae direct in a plankton sample

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Chapter 1

Population dynamics of the invasive Pacific oyster *Crassostrea gigas* during the early stages of an outbreak in the Wadden Sea (Germany)

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Abstract

Since the late 1990's, the Pacific oyster (*Crassostrea gigas*) has spread into the East Frisian Wadden Sea (Germany). This invasion provided an opportunity to study the population dynamics and the patterns of spread during the initial bioinvasion process. With its source area in The Netherlands, the bioinvasion continues in an eastward direction, as documented by a gradient of high abundances in the west and low abundances in the east during the first study year. One year later, abundances of the Pacific oyster were more heterogenic and differed between adjacent tidal basins. The increase in population sizes at all study sites was very high, reaching levels similar to native occurrence populations. The growth constant (*K*) varied between 0.300 y^{-1} and 0.990 y^{-1}. The mussel bed with the highest densities had a mean abundance of > 300 ind. m^{-2}, and a maximum of 1460 ind. m^{-2}. Furthermore, the bioinvasion was facilitated by a low mortality (*Z*) found for populations between half and one and a half years old (Z = 0.03 y^{-1} to 0.13 y^{-1}). At present, Pacific oysters are well established at several locations in the East Frisian Wadden Sea and may become with these reproductive potential self-sustaining populations.

Keywords

bioinvasion, population growth, mortality, larvae dispersal, non-indigenous

Introduction

Biological invasions consist of human induced species translocation such as global shipping and aquaculture (Carlton 1985, Gollasch 2002, Nehring & Leuchs 1999), or of natural range expansions (Carlton 1989). Once an alien species has arrived in a new region, further spread can contribute to the influence on the recipient region (Grosholz 2002, Wasson et al. 2001). The accelerated spread (worldwide and local) of non-indigenous species is also facilitated by climate change (Stachowicz et al. 2002, Walther et al. 2002). Understanding the dynamics of transport, settlement and invasion of non-indigenous species is therefore necessary to assess the long term consequences for marine ecosystems (Occhipinti-Ambrogi 2007). Particularly, the dynamic of an invasion during the initial phase of a successful invader could help to understand the mechanisms which invasion are underlying (see Grosholz 2002, Occhipinti-Ambrogi 2007). In this paper we describe the population dynamics of the invasive Pacific oyster (*Crassostrea gigas*; Thunberg, 1793) during their initial spread in the East Frisian Wadden Sea area of the North Sea.

The Pacific oyster is one example of a species that was intentionally for (e.g. aquaculture) or unintentionally introduced in many different regions, where it further dispersed by natural means (Andrews 1980, Chew 1990, Ruesink et al. 2005), e.g. North America (Andrews 1979, Quayle 1969), South America (Escapa et al. 2004), South Africa (Robinson et al. 2005), Australia (Ayres 1991, Dix 1991) and Europe: France (Grizel & Héral 1991), The Netherlands (Drinkwaard 1999), Germany (Reise 1998, Wehrmann et al. 2000) and England (Eno et al. 1997). An overview on bioinvasion by oysters is found in Ruesink et al. (2005).

In the southern North Sea, the Pacific oyster was deliberately introduced for aquaculture into the Oosterschelde (The Netherlands) in 1965 (Drinkwaard 1999, Reise 1998), the first successful spat falls of the Pacific oyster outside aquaculture plots occurred in 1975 and 1976 (Drinkwaard 1999). After these recruitment events, abundances increased along the southern North Sea coast. In 1983, Bruins (1983) reported the first Pacific oyster findings in the Wadden Sea attached to stones at Texel, and in 1998, Tydeman (1999) discovered Pacific

oysters in the Harbour of Eemshaven (at the Dutch-German border). On the East Frisian coast, the first individuals were found in 1998 (Wehrmann et al. 2000).

The spread of the Pacific oyster in the East Frisian Wadden Sea can be traced back to this earlier introduction to the Oosterschelde in The Netherlands (Wehrmann et al. 2000). Former failed attempts of introduction for aquaculture in the East Frisian Wadden Sea (dates and place of Pacific oyster aquaculture attempts: 1974 Neuharlingersiel; 1976 / 1982 Jade, 1982 Wangerooge, 1987 Norderney (Neudecker 1985, Wehrmann et al. 2000)) have not lead to the establishment of feral oysters in the area. A successful Pacific oysters aquaculture on the German coast is located at the island of Sylt, from where a spread throughout the northern Wadden Sea started five years after the introduction in 1986 (Diederich 2005, Nehls et al. 2006, Reise 1998). Although in the northern Wadden Sea attempts of introduction for aquaculture were done before the successful aquaculture at Sylt (see Nehls & Büttger 2007).

Previously, non-indigenous bivalves, such as the clam *Mya arenaria* (Strasser 1999) and the razor clam *Ensis americanus* (Armonies & Reise 1999), found a niche in the Wadden Sea ecosystem (see also Reise et al. 2005). A high “niche opportunity”, that appears to be provided by the Wadden Sea ecosystem raises the receptiveness of a community to invasive species. The “niche opportunity” defines conditions that promote invasions in terms of resources, natural enemies, the physical environment, interactions between these factors, and the manner in which they vary in time and space (Shea & Chesson 2002). The low species richness in the coastal northern European waters and the vacant ecological niches in the North Sea after the last glaciations can facilitate the success of invading species on the German coast (see Reise et al. 2006, Vermeij 1991, Kennedy et al. 2002, Levine & D'Antonio 1999, Levine 2000). Furthermore, as the Wadden Sea is a highly dynamic ecosystem (Reise et al. 2005), where e.g. sediment rearrangements may produce empty patches, it is very receptive to introduced species (Carlton 1996, Sousa 2001). However, unlike previous invasions by *M. arenaria* and *E. americanus*, the Pacific oysters are more likely to change the habitat structure in the Wadden Sea, affecting blue mussel beds (*Mytilus edulis*) and their associated organisms.

A remarkable increase in oyster abundance has already been observed in the Dutch and northern German Wadden Sea (Dankers et al. 2004, Diederich et al. 2005, Dankers et al. 2006), and was postulated to be due to low mortality and high growth rates in settled Pacific oysters during their first five years of age (Diederich et al. 2005, Diederich 2006, Reise 1998).

Our study is, to our knowledge, the first one documenting a marine bioinvasion during the first years in which populations of the invasive species about to arise. The beginning spread of the Pacific oyster in the East Frisian Wadden Sea (Wehrmann et al. 2000) provided the opportunity to study the population dynamic of the invader during the initial phase of the invasion. Especially from the east part of the East Frisian Wadden Sea no reports of Pacific oyster occurrences were known before our study. Our investigations concentrated on population growth and mortality of the Pacific oyster. For this purpose, densities and size-frequencies of Pacific oysters were investigated on tidal flats of the East Frisian Wadden Sea (Germany).

Methods

Study site

The study area covered the entire Wadden Sea of Lower Saxony (Germany) between the Ems estuary in the west and the Elbe estuary in the east (6°40' E to 8°40' E and 54°60' N to 53°20' N). The area is characterised by muddy to sandy tidal flats with a semidiurnal tide cycle and a tidal range of 2.3 m to 3.9 m. In the area between the mainland and the barrier islands, 15 blue mussel (*Mytilus edulis*) beds were chosen for the investigation (Figure 1), based on information provided by the National Park administration about the occurrence of Blue mussel beds, logistic considerations and similar conditions (such as flooding time, location in lower intertidal, similar high (approximately 0 m to 1.2 m above low tide). The mussel beds were also selected to assure an even distribution of study sites throughout the entire area. The investigation was carried out on blue mussel beds, as they provide the main hard substrate available for Pacific oysters settling in the Wadden Sea. Other hard substrates are shell beds, harbour walls, groins, dikes and other artificial substrates.

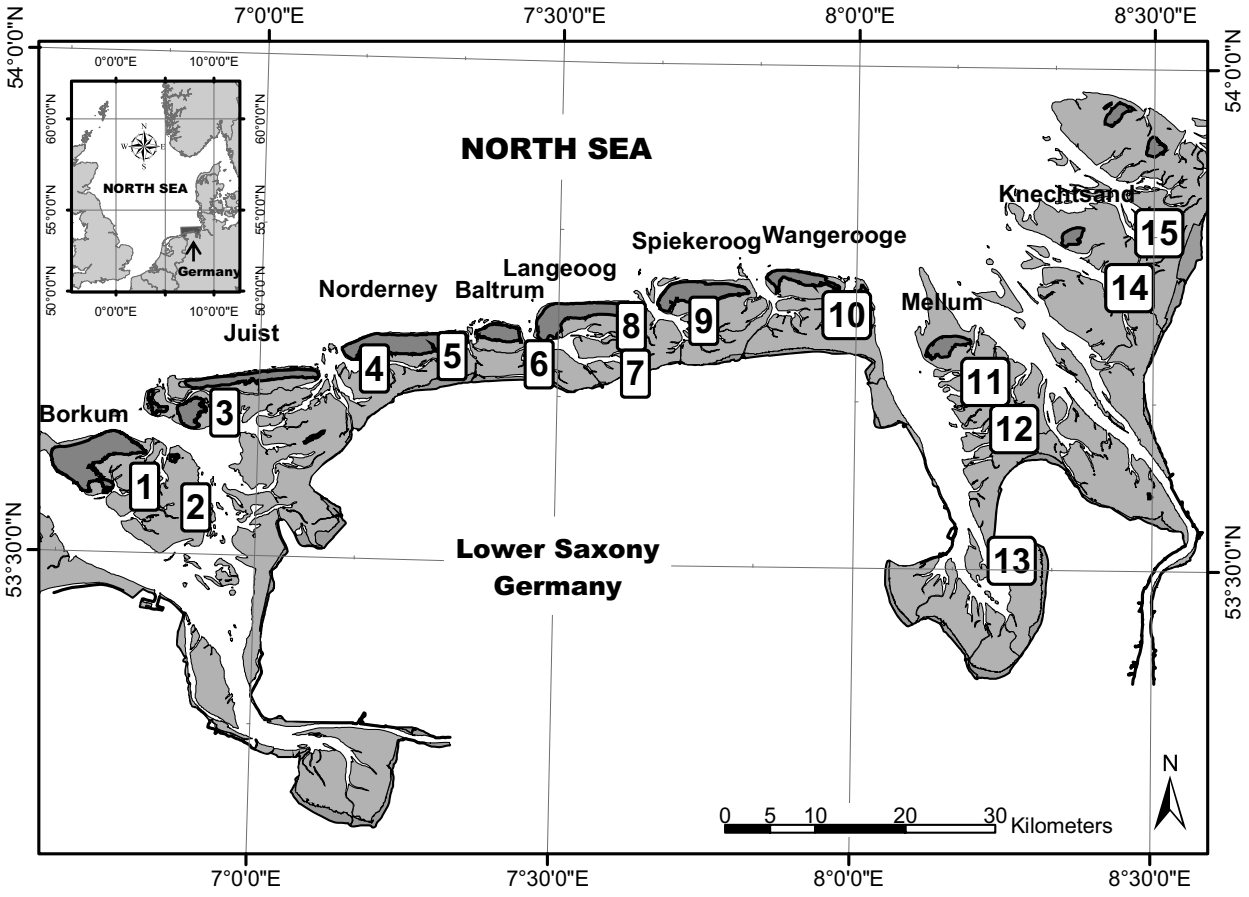

Figure 1: Locations of the 15 investigated mussel beds on the tidal flats of the Lower Saxony Wadden Sea, Germany. Shaded (light grey) areas indicate tidal flats.

The areas of the mussel beds were mapped using a global-positioning system (GPS). To assess the spatial extent of a mussel bed, all areas occupied by mussels not more than 25 m apart were included. These criteria were adapted from the blue mussel monitoring carried out by the National Park administration (see also Herlyn 2005). The area of a mussel bed can be separated in areas of patches were the bivalves are accumulated (in the beginning of the study also for the most part without oysters) and in patches with bare sand and mud which include scattered occurrence of bivalve shells, mussels and oysters. Additional investigations (e.g. shell growth) were carried out on four of the 15 mussel beds (#2, #3, #6 and #12). These four exemplary mussel beds were selected because: Mussel bed #3 and #12 differed in the first year of the investigation in their oyster abundance (#2 and #3 with high and #6 and #12 with low oyster abundance). Mussel bed #12 was easily accessible, mussel bed #2 had the

largest oyster population of the 15 mussel beds in the first year and mussel bed #6 closes the spatial gap between mussel bed #3 and #12 for an equal distribution of the additional investigations.

Annual Monitoring

Field surveys for estimate stock abundance of Pacific oyster were carried out in spring 2003, 2004, and 2005, before the spat fall of the respective year took place. A possible overlap of survey and spat fall occurred only in the first year, when samples were taken in September and October on mussel beds #4, #5, #6 and #9, which could increase the abundance of the young oysters.

A grid with 0°0.01' intervals was assigned over the whole area of each mussel bed and coordinates for 100 sampling sites allocated with a random number generator. As the mussel beds exist of patches of Blue mussel with or without Pacific oysters as well as sandy to muddy areas with more or less occasional occurrence of bivalve shells, single Blue mussels and Pacific oysters, the randomly distributed sampling grids included the range of microhabitat topography of a mussel bed. The sampling sites were located by using a GPS (Garmin GPS 72) and were marked with bamboo sticks for relocation in the following years. At each sampling site, oyster density was determined within a 1 m^2 quadrate and the size of oysters measured as the largest distance from the hinge, using a calliper to the nearest mm. Measurements were done in the field, and all oysters were left on the sampling site, to avoid a change in the natural composition. From 2004 onward, the quadrate size was reduced to 0.25 m^2 for those mussel beds showing a high abundance of Pacific oysters. Oyster densities are given as mean abundance ± standard error (SE) of the 100 sampling sites per mussel bed for the comparison of the abundance increase between years. For the data analysis, the abundance of the mussel beds (each mussel bed n=100) was used, but in Figure 2 abundance data are plotted as Box & Whisker-Plot to better reflect the patchiness on a mussel bed. Variations were high as a mussel bed comprises areas occupied by mussels and sandy or muddy open space in between. The maxima are representing oyster patches on a mussel bed.

Data analysis

Abundance distributions of the Pacific oyster in the entire study area

The annual population increase is calculated from mean oyster abundances of all investigated mussel beds. Data were tested for normality using the Kolmogorov-Smirnov-Test, but as assumptions for ANOVA could not be met, non-parametric statistics applied. To test for inter annual changes in abundance we used the Wilcoxon signed-ranks test or the Friedman test. To compare the Pacific oyster populations between the 25 mussel beds for each year separately, the Kruskal-Wallis-H-Test was used. As post hoc analysis, the Nemenyi-Test was used. Effects were considered to be statistically significant if the P-value was ≤0.05. The year to year increase was calculated for the mean of all mussel beds.

Length frequency distribution, population growth and mortality

Length frequency distributions were determined for all investigated mussel beds, but are presented only for the exemplary mussel beds: #2, #3, #6 and #12 (5 mm size classes, annual). For a better comparison of the length frequency distribution between the mussel beds, the data are shown as relative abundances. The length frequency distribution data were fitted to the “von Bertalanffy growth function” (VBGF) with the program FiSAT II. This was done for all mussel beds, except for the mussel beds #4, #10, #13 and #15 where calculations were impossible due to low population size. For the calculation of the growth constant (K) of the VBGF, the Shepherd's method from the FiSAT II was used. The length L_∞ is given from FiSAT II and represents the largest accepted length present in the population. The possible maximum length of the Pacific oyster was not reached during the studied period of early invasion, therefore we used L_∞ given by FiSAT II. The mortality rate Z y^{-1} for the entire duration of the investigation was calculated by a fit of the size converted catch curve with FiSAT II. Additionally, the year to year mortality was calculated on the exemplary mussel beds with a high oyster abundance (#2 and #3) by direct determination over the cohort size with the formula:

$$-Z = \ln\left(\frac{N_1}{N_0}\right)$$

Where *Z* is the mortality, ln the natural Logarithm, N_1 the cohort size at time T_1 and N_0 the cohort size at T_0. These calculations do not include the early mortality directly after settlement due to the mode of data collection. The abundance and length of the N_0 generation was determined approximately half year after settlement, so the first generation is further noted as N_1 generation. The cohorts, size and mean length for the calculation of mortality and mean shell growth was determined with the Bhattacharya's method using FiSAT II.

The mean shell growth in the second and third year was calculated from the differences between the mean lengths of single cohorts from the mussel bed #3 and #12.

Results

Abundance and distribution of Pacific oyster in the East Frisian Wadden Sea

In the course of the three study years (2003 – 2005), an increasing spread of Pacific oyster was recorded from the west towards the east. The quantitative surveys on 15 mussel beds throughout the study area demonstrated this population increase (Figure 2). The abundances (ind. m^{-2}) of the Pacific oysters differed significantly on the mussel beds over the years (Table 1). In 2003, three groups with similar oyster abundance each and a significant distinction between the groups ($p \leq 0.05$) were distinguished after the comparison of all mussel beds (group 1 with mussel bed #1 - #3; group 2 with #4 - #7 and group 3 with #8 - #15; see Figure 2, year 2003). These groups represented mussel beds with similar oyster densities descending in densities from the west (high) to the east (low), but in 2004 and 2005 this spatial distribution exist not any more. This and the similarity between mussel beds in each year is visible in Figure 2, especially the maxima showing the similarity because they indicate that on the patchy mussel beds some areas had a similar high oyster density.

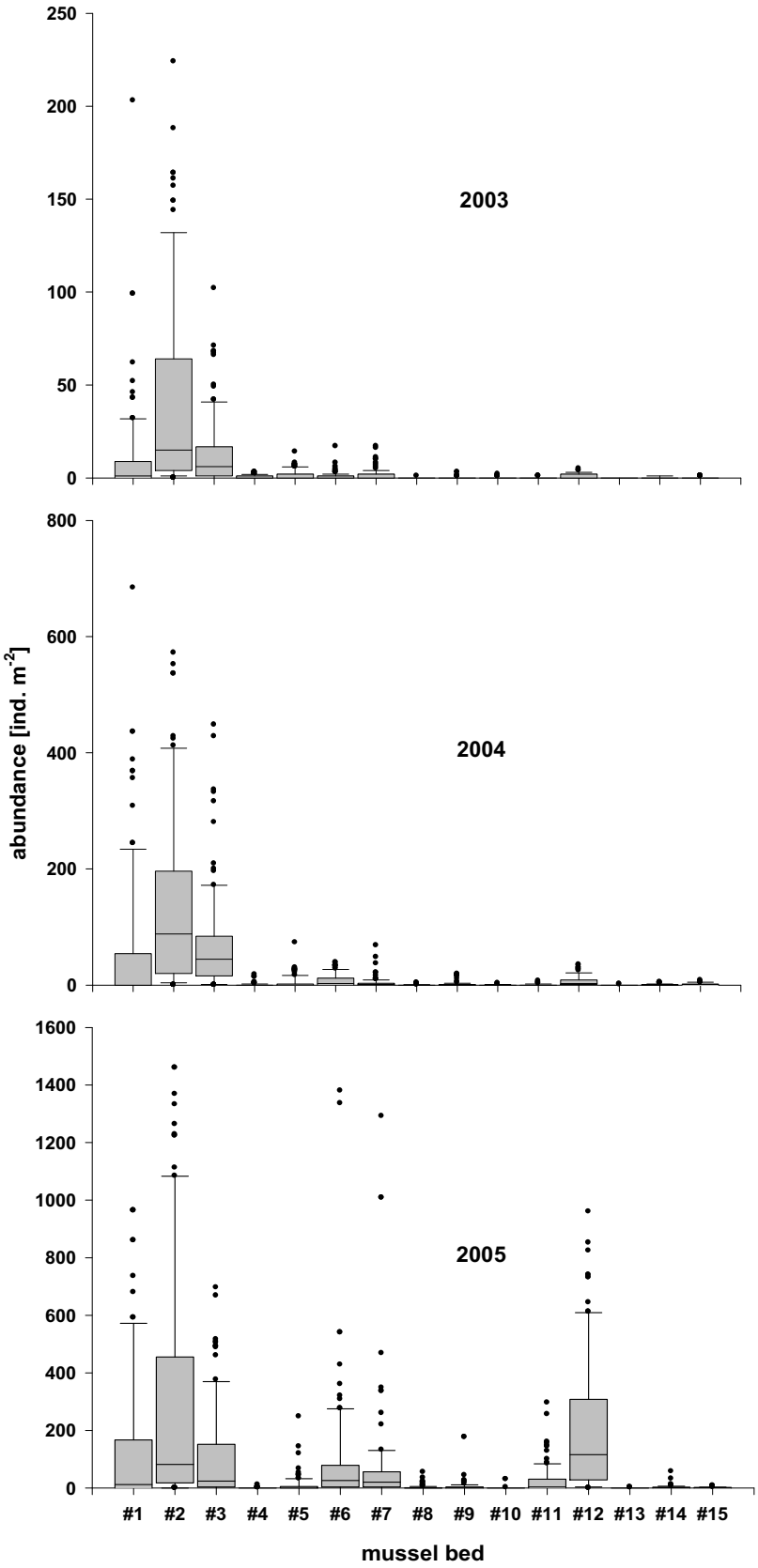

Figure 2: Abundance of the Pacific oyster on all investigated mussel beds (for the location see Figure 1) in the years 2003, 2004 and 2005. Box plots show median (horizontal line within box), 25th and 75th percentiles (box) and 10th and 90th percentile (whiskers); circles indicate all outlier. For the comparison of abundances during one year each Box & Whisker-Plot has different scaled axis of abscissa.

Table 1: Spatial extent of all investigated mussel beds, with mean abundance ± SE of Pacific oyster in 2003, 2004 and 2005

Mussel bed	Mussel bed area 2003 [ha]	Crassostrea gigas (ind./m2)			Significant change of the abundance
		2003	2004	2005	
		mean ± SE	mean ± SE	mean ± SE	
# 1	3.13	11.11 ± 2.65	55.40 ± 12.05	150.56 ± 25.71	a, b, c
# 2	22.83	42.44 ± 5.34	136.24 ± 14.90	302.04 ± 40.96	a, b, c
# 3	37.09	13.32 ± 1.92	70.92 ± 8.92	107.97 ± 16.12	a, b, c
# 4	7.67	0.41 ± 0.08	0.83 ± 0.27	0.23 ± 0.12	a, c
# 5	18.98	1.47 ± 0.25	4.22 ± 1.02	10.73 ± 3.24	a, b, c
# 6	11.09	0.96 ± 0.21	7.99 ± 1.05	93.80 ± 21.16	a, b, c
# 7	28.54	1.59 ± 0.31	3.91 ± 0.94	77.36 ± 19.94	a, b, c
# 8	48.85	0.01 ± 0.01	0.18 ± 0.18	2.42 ± 0.74	a, b, c
# 9	28.69	0.12 ± 0.05	1.66 ± 1.66	6.47 ± 2.53	a, b, c
# 10	6.77	0.09 ± 0.03	0.2 ± 0.05	0.62 ± 0.42	a, b, c
# 11	14.21	0.04 ± 0.02	0.46 ± 0.11	27.85 ± 5.20	a, b, c
# 12	6.51	0.91 ± 0.12	6.67 ± 0.85	205.56 ± 23.61	a, b, c
# 13	14.15	0	0.03 ± 0.02	0.06 ± 0.04	
# 14	5.91	0.13 ± 0.03	0.67 ± 0.11	2.58 ± 0.67	a, b, c
# 15	12.29	0.09 ± 0.03	1.42 ± 0.18	1.24 ± 0.16	a, b

Significant increase is shown ($P \leq 0.05$): a = Friedman-test (all 3 years of investigation), b and c = Wilcoxon-test (b = 2003–2004) (c = 2004–2005). At mussel bed #4 the change between 2004 and 2005 was a significant decrease.

In 2003, the largest oyster populations were found in the west, south of the islands Borkum and Juist, with mean abundances of 11.11 ind. m^{-2} (mussel bed #1) up to 42.44 ind. m^{-2} (mussel bed #2) and a maximum on a sample plot of 224 ind. m^{-2} (mussel bed #2). Mean abundances were lower towards the eastern parts and did not exceed 1.59 ind. m^{-2}, with no Pacific oysters found in the Jade Bay (mussel bed #13).

In 2004, a significant increase of the Pacific oyster abundances occurred on 13 of the 15 mussel beds (Table 1), and the first oysters were found in the Jade Bay. Oyster abundances increased 5.3 times in the whole study area from 2003 to 2004. The oyster densities of the mussel beds #5, #6, #7 and #12 were significantly higher (Table 1), with a four to eight times increase in comparison to the adjacent tidal basins. The highest mean abundance of 136.24 ind. m^{-2} was found on mussel bed #2. The maximum abundance on a sample plot recorded in 2004 was also in the western region on mussel bed #1 with 684 ind. m^{-2}.

By 2005, the Pacific oyster population had increased further, with an overall increase of 9 times over 2004 for the whole study area. The increase was highest on mussel beds #6, #7 and #12, where abundances reached the level of the mussel beds in the west. The largest population was still on mussel bed #2 with a mean abundance of 302.04 ind. m^{-2} and a maximum oyster number found on a sample plot of 1460 ind. m^{-2}. On mussel bed #4 the abundance of the Pacific oyster decreased significantly, because this and also mussel bed #15 were almost destroyed during the winter 2004 - 2005.

Length-Frequency-Distributions

Oysters found ranged in size from 1 mm to 234 mm. On the basis of Length-Frequency-Distributions (Figure 3), one new cohort was identified by FiSAT II in every year. The N_1 cohort of the year 2003 (recruits of autumn 2002 which were already approximate half a year old) could be followed up to the N_3 generation. The N_1 and N_2 cohorts were visible as bimodal peaks in 2004 and 2005.

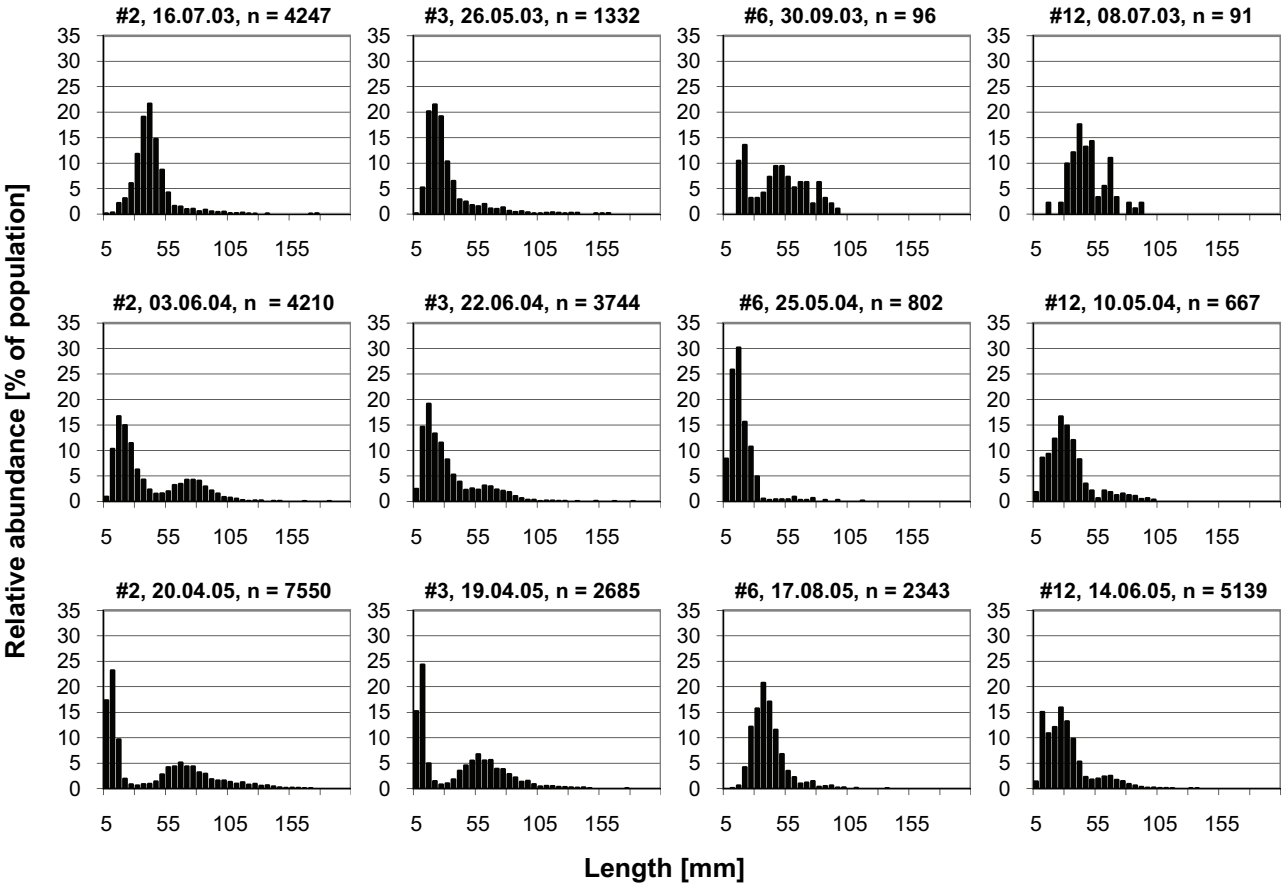

Figure: 3 Annual length-frequency distribution (LFD) from Pacific oysters of four selected mussel beds (mussel bed #2, #3 and #12) in 2003, 2004 and 2005

For the second and the third year, the annual shell growth was calculated from the Length-Frequency-Distribution of mussel bed #3, #6 and #12, for example. At mussel bed #3, the mean growth of the Pacific oyster was 44 mm y^{-1} in the second year of growth, and 27 mm y^{-1} in the third year. For mussel bed #12, the Pacific oyster grew 29 mm y^{-1} on average in their second year and 32 mm y^{-1} in the third year. For calculations on mussel bed #6 only the last two years could be used, with a growth of 46 mm y^{-1} for the second year and 40 mm y^{-1} for the third year.

To describe the population growth and mortality over three years, the VBGF were fitted to the data sets and the growth constant (K) with the maximal expected length (L_∞), and the mortality (Z) was calculated. The growth constant of the VBGF varied in the range from K = 0.300 y^{-1} at mussel bed #3 with L_∞ = 186.90 mm and K = 0.990 y^{-1} at mussel bed #11 with L_∞ = 129.15 mm (Table 2).

Table 2: L_∞, growth constant (K) and mortality (Z) of the Pacific Oyster populations calculated for the 3 years of investigation

Mussel bed	L_∞ (mm)	$K\ y^{-1}$	$Z\ y^{-1}$
#1	207.90	0.440	1.55
#2	192.15	0.390	1.41
#3	186.90	0.300	1.19
#5	144.90	0.410	1.20
#6	139.65	0.580	1.67
#7	160.65	0.910	2.92
#8	139.65	0.320	0.89
#9	139.65	0.510	1.44
#11	129.15	0.990	3.16
#12	139.65	0.580	2.14
#14	92.40	0.670	1.53

For mussel beds #2 and #3, the mortality (Z) was calculated for the 2003 N_1 cohort. Between a half and one and a half year, the mortality on mussel bed #2 was Z = 0.03 y^{-1} and on #3 Z = 0.13 y^{-1}, after one more year the mortality on mussel bed #2 was Z = 0.82 y^{-1} an on #3 Z = 1.61 y^{-1}. The mortality rate Z y^{-1} calculated with FiSAT II for the entire duration of the investigation was in the range of 1.19 y^{-1} to 3.16 y^{-1} (Table 2) and therefore many times higher than the calculations for the first and second year were showing.

Discussion

Investigations describing the population dynamics of a beginning marine bioinvasion are rarely available in the literature. Most studies describe the current state of a bioinvasion (e.g. Grizel & Héral 1991, Mann &Harding 2000, Streftaris et al. 2005) or the change over several years (e.g. Diederich et al. 2005, Escapa et al. 2004, Herkül et al. 2006, Oliveira et al. 2006). The first investigation on Pacific oyster appearance outside of the 1986 established oyster farm at the island Sylt situated in the northern part of the German Wadden Sea were done by Reise (1998), he described the oyster occurrence around the island in 1993 and 1996, while the first findings of the Pacific oyster in the East Frisian Wadden Sea were done in 1998 by Wehrmann et al. (2000). These findings in the East Frisian Wadden Sea provided the opportunity for this case study to examine the beginning of a bioinvasion and the beginning spread of a marine invasive species on a large scale of about 120 km of coastline.

The invasion of the Pacific oyster, starting from the aquacultures in the Oosterschelde, is only partly described for the Dutch Wadden Sea (Dankers et al. 2004, Drinkwaard 1999, Tydeman 1999) and the East Frisian Wadden Sea (Wehrmann et al. 2000), while more is known about the oyster population in the northern German Wadden Sea (Diederich et al. 2005, Diederich 2006). Both invasions differ in the origin of the non-indigenous oyster. In the East Friesian Wadden Sea the invasion of Pacific oyster is a spread by natural means with a distant introduction source (Wehrmann et al. 2000), whereas the invasion in the northern German Wadden Sea is enforced by a continuous input of larvae from a local oyster farm (Diederich et al. 2005). It is important to differentiate both invasion events to understand the causes for their successful bioinvasion.

The successful introduction of an invasive species is divided into four successive phases: arrival, settlement, expansion and persistence (Mollison 1986, Reise et al. 2006). For the invasion of the Pacific oyster in the East Frisian Wadden Sea we could detect the first three phases of introduction. The arrival of the Pacific oyster in the western part of the east Frisian Wadden Sea was postulated to have been taken place between 1994 and 1996 (Wehrmann et al. 2000), and the first discovery in the eastern part of the east Frisian Wadden Sea was in

2001 (Herlyn & Millat 2004). The first small populations in the west were found in 1998/1999 (Wehrmann et al. 2000) and in the east in 2002 / 2003 (Herlyn & Millat 2004, and this study). With the spat fall from 2002, the population in the west started the expansion phase with a significant increase in abundance (Table 1), and the same was observed in 2005 in the east. Hence, the oyster had an establishment phase of 4 to 6 years. For the oysters which were introduced in 1986 in the List tidal basin at the island Sylt, the first significant spatfall was in 1991 (Diederich et al. 2005, Reise 1998), five years after introduction. This is in agreement with the expected establishment phase of four to six years for a Pacific oyster population in the southern North Sea. After the establishment phase, only one or two more generations are necessary for a rapid increase of the population. Our investigations started with the beginning expansion phase of the invasive oyster in the East Frisian Wadden Sea. The population increase differed between mussel beds. We found that the population increase was faster in the vicinity of the accepted local source populations than further away, e.g. mussel bed #6, #7 had a higher increase than the nearby beds #5 and #8 located in the adjacent tidal basins and mussel bed #12 in comparison to nearby bed #11 (see Figure 2). Similar effects were described by Diederich et al. (2005) for the List tidal basin at the island of Sylt. This local increase we found, with a successful recruitment in every investigated year indicates that the spreading of the oyster takes place in a restricted area around the source population. A possible cause for the local increase could be the hold off storm events resulting in lacks of water currents, therefore without storm events larvae are retained in a restricted area around the source population (see also Dunstan & Bax 2007).

Key factors for the spread are the wind conditions together with a successful spat fall, as shown for the invasive mussel *Mytilus galloprovincialis* in South Africa (McQuaid & Phillips 2000). Our study period fell into years with low mean wind speed during the spawning period (data Deutscher Wetter Dienst (DWD)). A natural wide spread dispersal eastwards of approximately 150 km per year of a benthos species with a free swimming larval phase of around 20 to 30 days is a theoretically expectable value under special weather conditions at the southern North Sea coast (Armonies 2001, Wehrmann et al. 2000). The spread is attributed to the eastward residual current of approximately 0.1 m s^{-1} due to special wind

conditions (constantly over a 3 - 4 week period) existing in coastal waters of the southern North Sea (Armonies 2001, Wehrmann et al. 2000). Wide dispersal of larvae only happens during such wind conditions, as was the supposed case for the spread of the oyster in the years 1994 to 1996 (Wehrmann et al. 2000). In these years, the oyster recruited on a coast line of approximately 50 km between the islands of Borkum and Baltrum on the East Frisian coast, coming from the western Netherlands as indicated by the comparison of oyster abundances in 2003, where we found decreasing abundances towards the east (see Figure 2). In 1994 and 1995 the water temperature was significantly higher during the oyster larvae period from July to October than average, coinciding with a significant oyster recruitment in 1994 at the island of Sylt (Diederich et al. 2005), and also the wind was significantly stronger than average in the period between 1990 and 2005, with a mean south western wind direction (DWD), both factors had a positive influence for an eastward wide spread of oyster larvae. Additional to dispersal by current, we are not able to exclude the possibility that the oyster was further transported within the Wadden Sea by human activities such as ship transport, whether on the hull or in ballast water (Carlton 1999). Chew (1990) described the introduction of the Pacific oyster to New Zealand by the arrival of adult oysters on hulls of ships with a subsequent settlement, prosper and reproduction of their spawn. One hint for additional dispersal of the oyster by ships comes from the massive spat fall in 1999 in the harbour of Eemshaven (The Netherlands) (Tydeman 1999).

Most studies on the population dynamics of marine invertebrates are carried out for non invasive populations, for example the bivalves *Aequipecten opercularis* (L.) from the western English Channel (Heilmayer et al. 2004), *Donax serra* from Namibian sandy beaches (Laudien et al. 2003) and from the Wadden Sea *Cerastoderma edule* (Ramón 2003) and *Mytilus edulis* (Munch-Petersen & Kristensen 2001). Only the studies of Diederich (Diederich et al. 2005, Diederich 2006) give the opportunity for a detailed comparison of the population dynamic of the invasive Pacific oyster in the East Frisian Wadden Sea.

The population growth rate (the constant (*K*) from the von Bertalanffy growth function (VBGF)) we calculated for the East Frisian Pacific oyster population was threefold higher than that calculated by Diederich (2006) of the North Frisian population.

In comparison to the above mentioned non invasive populations, the population growth constant of the Pacific oyster from the Wadden Sea (range K = 0.3 – 0.99 y^{-1}) is in the range of native natural occurrence populations: K = 0.604 y^{-1} for the population of *Aequipecten opercularis* (L.) from the western English Channel (Heilmayer et al. 2004), K = 0.274 y^{-1} for the population of *Donax serra* from Namibian sandy beaches (Laudien et al. 2003) and K = 0.404 y^{-1} for the *Cerastoderma edule* population from the Wadden Sea (Ramón 2003) and K = 0.243 y^{-1} - 0.902 y^{-1} for the *Mytilus edulis* population (Munch-Petersen & Kristensen 2001). Thus, the Pacific oyster population in the Wadden Sea can be described as a population which can be viable.

The formation of Pacific oyster populations in different parts of the world show how successful the oyster is as an invader. In Chile, the first invasive oysters were found five years after introduction in 1982, then from 1995 there was incipient colonization with 2 oysters m^{-2}; settlement increased explosively since 1998 with a density of up to 120 recruits m^{-2} (Orensanz et al. 2002). On South African shores, the Pacific oyster had a relatively long establishment phase of 51 years. It was introduced in the 1950s and wild oyster populations were first recorded in 2001 (Robinson et al. 2005). Even though the time was very long between the oyster introductions and the first wild population, the oyster was able to persist and adapt to the new environment where it could build up a stable population in the end. The establishment phase we found, similar to the invasion in Chile and that point that the oyster can build viable populations even after a very long time after introductions like in South Africa together with the considerable increase of the Pacific oyster abundance indicates the possibility that an self sustaining populations com into begin in the Wadden Sea.

The success of the Pacific oyster invasion in the East Frisian Wadden Sea was further accelerated by the low mortality of half to two years old juveniles. For the young oyster population of the north Frisian Wadden Sea, Reise (1998) also reported a low mortality, in spite of a foregoing severe winter. Calculated (with FiSAT II) over the three years of our investigation, total mortality was Z = 1.19 y^{-1} - 3.16 y^{-1} and thus in the range of other mussel populations in the Wadden Sea, e.g. *Cerastoderma edule* with Z = 0.52 y^{-1} - 3.03 y^{-1} (Ramón 2003) and *Mytilus edulis* with a mean annual mortality of Z = 0.84 y^{-1} (Munch-Petersen &

Kristensen 2001). In contrast to the higher mortality of *M. edulis* during the first years, the mortality of the oyster population we found increased with the years. Also Diederich (2006) found low mortality rate for the first three months post-settlement (M = 0.004 d^{-1}) and during the first winter (M = 0.005 d^{-1}) of the Pacific oyster during experiments in the List tidal basin near the island Sylt. The low mortality we found can indicate a lack of oyster predators during the first period of the bioinvasion. Main predators like the shore crabs *Carcinus maenas* and the starfish *Asterias rubens* prefer the native blue mussel over the non-native Pacific oyster (Diederich 2005). After several years, predators could learn to feed on the non native oysters and the mortality in the first year would increase.

The analysis of the length-frequency-distribution showed a new cohort in every investigated year on each mussel bed. A successful recruitment was also observed for the years 2001 to 2003 for the northern area of the Wadden Sea of Germany (Diederich et al. 2005). The successful recruitments can be related to warm summers with above-average temperatures of more than 18°C in July / August (Diederich et al. 2005). Therefore, the spread of the oyster in the Wadden Sea seems to have benefited from temperature rise with climate change, which is also affecting native bivalves in the Wadden Sea (Beukema and Dekker 2005).

The individual mean growth we calculated for the oysters of the East Frisian population was similar to the growth of the oysters in the northern part of the Wadden Sea, where Diederich (2006) described the individual growth rate as somewhat lower compared to other areas and only slightly lower than in the native habitat (Japan and Korea). The high individual growth rate can protect the oyster against predation and can function as protection against the environment. On a mussel bed which accumulated a lot of sediment we observed oysters growing with a long and thin shell, whereas on another mussel bed, which was exposed more against waves, the oysters produced thicker shells.

The now widespread occurrence and high abundance of the invasive Pacific oyster in the East Frisian Wadden Sea suggests likely effects of this introduced bivalve for the Wadden Sea ecosystem, e.g. a substitution of blue mussel beds with oyster reefs or a change to mixed beds of Pacific oysters and Blue mussels. In many other regions, introduced molluscs are now

the most abundant infaunal or epifaunal species (Carlton 1999); e.g. *Mytilus galloprovincialis* is the dominating mussel throughout the Western Cape region of South Africa, where they largely displaced the native mussel *Aulacomya ater* (Griffith et al. 1992). Considering the current population growth and low mortality, our investigation allows the prediction that the Pacific oyster presently has the potential to become one of the most abundant epifaunal bivalves in the Wadden Sea.

Acknowledgments

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Chapter 2

Comparison of the population dynamics of the invasive Pacific oyster (*Crassostrea gigas*) with the native Blue mussel (*Mytilus edulis*) in the Wadden Sea (Germany)

Submitted:

Schmidt A, Herlyn M, Millat G, Wehrmann A, Dittmann S (Submitted) Comparison of the population dynamics of the invasive Pacific oyster (*Crassostrea gigas*) with the native Blue mussel (*Mytilus edulis*) in the Wadden Sea (Germany). Biol. Inv.

Abstract

Successful bio-invasions have an impact on the native ecosystem, but the reasons for the success of a non-indigenous species in the new environment remain often unclear and the ultimate causes of an impact are often unknown. We investigated the influence of an increasing population of introduced Pacific oyster (*Crassostrea gigas*) on the native Blue mussel (*Mytilus edulis*) population in the East Frisian Wadden Sea (Germany). A comparison of the population dynamics of the oyster and mussel indicated that the establishment of Pacific oysters had no negative effects on the Blue mussel population, yet, although the oyster had a fivefold higher biomass production than the Blue mussel. The high biomass supplied by the oyster could have an influence on the food web. We further investigated factors that can influence the success of the oysters, such as temperature and settlement substrate. Water temperatures necessary for oyster reproduction were reached in the three consecutive years of this study. The oyster larvae settled preferentially on con-specific shells, which may enhance the establishment of oyster reefs. Oyster reefs gave shelter to the Blue mussel (as well as to other species) due to their complex structure. While this study showed no apparent impact of the Pacific oyster on the Blue mussel population, the biomass production and consecutive successful recruitment of the Pacific oyster indicate further changes to the Wadden Sea ecosystem.

Keywords

Crassostrea gigas, *Mytilus edulis*, North Sea, bio-invasion, alien species, non-indigenous

Introduction

Worldwide, marine invasive species have been documented to impact the recipient region (Carlton 1996, Kotta et al. 2006, Mack et al. 2000, Ricciardi & Atkinson 2004). Yet, there still remain knowledge gaps why species become invaders and why certain species and not others succeed in a new region (Crooks 2002). It is therefore important to investigate invaders as well as the invaded respectively the donor community (Shea & Chesson 2002). Such information may allow the development of better prediction models if species are successful as invader and if they have an influence on the recipient region.

One essential criterion for a species to persist in a community is the ability to increase from low density, which is also an important condition for an alien to be able to invade and survive in a recipient community (Shea & Chesson 2002). The success of an aquatic invasive species is furthermore determined by a variety of attributes such as wide environmental tolerance, high genetic variability, short generation time, early sexual maturity, high reproductive capacity, and a broad diet (Essink & Dekker 2002). On community level, Ricciardi and Atkinson (2004) showed that high-impact invaders are more likely to belong to genera not already present in the invaded ecosystem. Furthermore, the possibility to reach environmental conditions allowing growth and reproduction is important to develop a self sustained population, and depends on factors such as food supply (Kang et al. 2000), temperature (Chávez-Villalba et al. 2002, Eno 1994, Ruiz et al. 1992), salinity (Kenny et al. 1990, Ortega & Sutherland 1992) and substrate (Lenihan 1999), which can further protect against predation (O'Beirn et al. 2000).

The Pacific oyster (*Crassostrea gigas*) invaded marine environments worldwide, which makes it an excellent example for the study of invader success and impacts on benthic communities.

Until 1964 the Pacific oyster did not exist in the North Sea Wadden Sea (Drinkwaard 1999). In former times the existing oyster species was the European oyster (*Ostrea edulis*), which was distributed mainly in the subtidal and is now extinct in the southern North Sea. The invading Pacific oyster is mainly colonising on Blue mussel beds in the tidal flats of the

Wadden Sea. Since the Pacific oyster invaded the Wadden Sea (1983 at Texel in The Netherlands (Bruins 1983); 1986 at Sylt in the north of Germany (Reise 1998); and 1998 in the East Frisian Wadden Sea (Wehrmann et al. 2000)), Blue mussel beds were increasingly occupied by oysters. Within the East Frisian Wadden Sea the population of the Pacific oyster had a reproduction rate of about 5.3 times from 2003 to 2004, and about 9.06 times from 2004 to 2005, respectively (Schmidt et al. 2008), whereas for the Blue mussel population of the East Frisian Wadden Sea a decrease was documented until 2003 (see Herlyn & Millat 2000, Herlyn & Millat 2004). The habitat shift from Blue mussel beds into oyster reefs has already occurred in the northern part of the German Wadden Sea around the island of Sylt (Diederich 2005a, Reise 1998) and in the Netherlands (Dankers et al. 2004).

The bioinvasion of the Pacific oyster in the Wadden Sea is supposedly facilitated by climate change, although the influence of water temperature on the oyster development and further species interactions are not entirely clear. A higher average water temperature in summer and mild winters in the past decade accelerated the success of the oyster invasion (Diederich et al. 2005), but inhibited mussel recruitment (Beukema et al. 2001, Diederich 2005a, Strasser et al. 2001). The high growth rate of oysters and high survival after cold winters secure the continued existence of the oyster population in years with low recruitment or environmental stress, e.g. through storms and ice scouring (Diederich 2006, Reise 1998).

The aims of our study were to investigate the influence of the increasing Pacific oyster population on the Wadden Sea ecosystem, especially on the Blue mussel population. Therefore we compared the population dynamics of the Pacific oysters and Blue mussels during the beginning increase of the Pacific oyster population. Furthermore we assessed the influence of the main abiotic factors, e.g. temperature and salinity, which may affect the development and growth of the Pacific oyster. In this context we analysed also the influence of settlement substrate for the spread of the oyster. This allowed us to evaluate possible future of a progressive increase in the Pacific oyster population on the Wadden Sea ecosystem.

Materials and Methods

Study site

The study area is located in the Wadden Sea of Lower Saxony (Germany), ranging from the Ems estuary in the west to the Elbe estuary in the east (6°40' E to 8°40' E and 54°60' N to 53°20' N) This area is characterised by muddy to sandy tidal flats with a semidiurnal tide cycle and a tidal range of 2.3 m to 3.9 m. The salinity in the near shore varied between 28.0 ‰ and 32.2 ‰ along the coast (own data). In the area between the mainland and the barrier islands, mussel beds were chosen where data for both species, Pacific oyster and Blue mussel, were available (#1, #3, #5, #8, #10, #11, #12, #14 and #15). These selected mussel beds belonged to 15 Blue mussel beds (Figure 1) which were already used for a Pacific oyster monitoring in the East Frisian Wadden Sea (for further details see Schmidt et al. 2008).

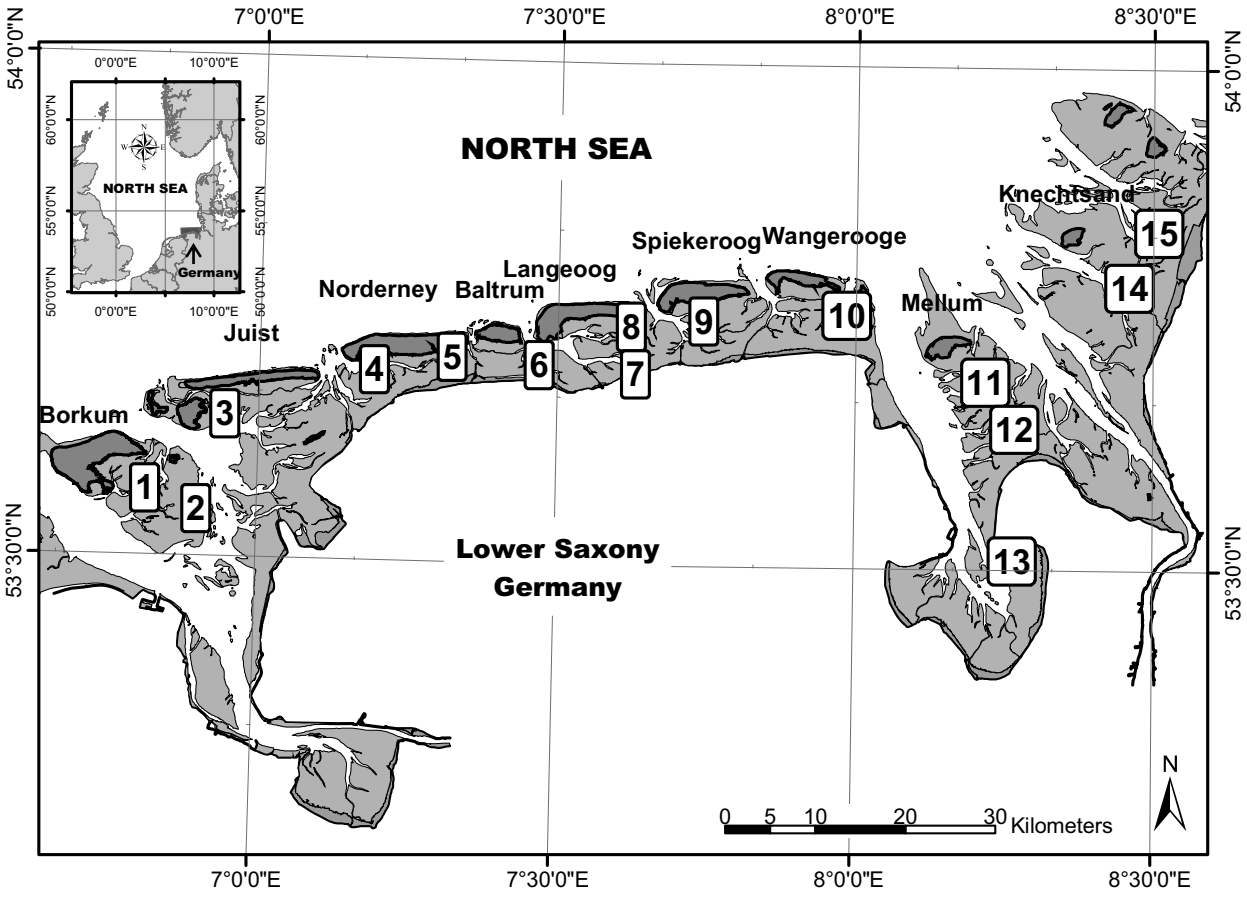

Figure 1: Map of locations of 15 for the Pacific oyster survey investigated mussel beds, on the tidal flats of the Lower Saxony Wadden Sea, Germany.

All investigations were carried out on Blue mussel beds, because they provide the main available hard substrate for Pacific oysters. Other hard substrates for oyster settlement include shell beds, harbour walls, groins, dikes and other artificial buildings (own observations).

The area of each mussel bed was mapped using a global-positioning system (GPS). To assess the spatial extent of a mussel bed, all areas occupied by mussels not more than 25 m apart were included. These criteria were adapted from the Blue mussel monitoring carried out by the National Park administration (see also Herlyn 2005).

In addition, biomass (#2, #3, and #12) and temperature (#3 and #12) data were collected on three selected mussel beds, because mussel bed #3 and #12 differed in oyster abundance in the first year of the investigation (#3 with high and #12 with low oyster abundance) and mussel bed #2 had the largest oyster stock of the 15 mussel beds in the first year. For the substrate preference analysis data of all 15 mussel beds were used.

Temperature measurement

Seabed temperature measurements were carried out on mussel bed #3 and #12. Temperature loggers (Tinytag Aquatic, Gemini Data Loggers (UK) Ltd.) were installed 5 cm above the ground of the mussel bed and were covered with a slab of plastic against direct insolation. Temperature measurements happened every 30 minutes, i.e. during high tide we measured the water temperature and during low tide the air temperature. Gaps in the measurement from October 2003 until March 2004 were due to weather conditions which made it impossible to readout the temperature logger.

Annual Monitoring

Pacific oyster monitoring

Field surveys to estimate the stock abundance of Pacific oysters were carried out in spring 2003-2005, before the spat fall of the respective year took place. A possible overlap of survey and spat fall occurred on mussel beds #4, #5, #6 and #9 only in the first year.

Over the whole area of each mussel bed, 100 sampling sites were randomly selected. At each sampling site, oyster density (within a 1 m^2 quadrate) and size were measured (largest

distance from the hinge, using a calliper to the nearest mm). In addition, the substrate on which each individual settled was determined. From 2004 onward, quadrate size had to be reduced to 0.25 m^2 for those mussel beds showing very high oyster abundances. Oyster densities are given as mean abundance per m^2 ± standard deviation (SD) of 100 sampling sites per mussel bed. The settlement substrates used by the oyster are expressed as percentage portion of all counted oysters from all examined mussel beds per year. Additionally, we determined the area covered by hard substrate (area in percent of the investigated area of all mussel beds) that is useable by the oyster as settlement substrate.

Blue mussel monitoring

Field surveys for estimating stock abundance of Blue mussel were carried out in spring 2003-2005. Over the entire mussel bed area 12 samples were randomly taken each year (177cm², core 15 cm in diameter). At each sampling site, mussel abundance was determined

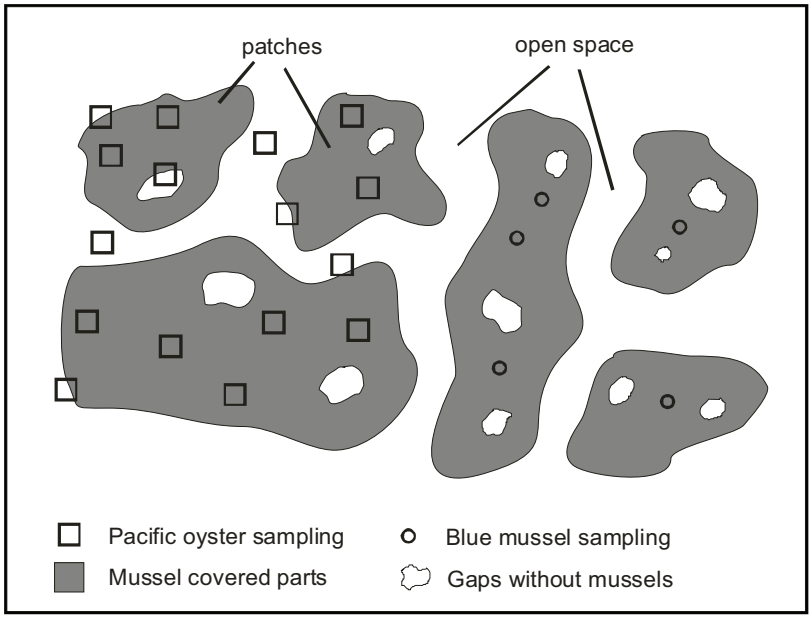

Figure 2: Schematic diagram of an intertidal mussel bed, which is divided in mussel covered patches and open spaces without mussels. The quadrates on the left demonstrated the sampling for the Pacific oyster monitoring, and the circles on the right the sampling for the Blue mussel monitoring.

and the size of the mussels was measured (as explained above). The sampling, different to that of the Pacific oyster, took place only on top of the Blue mussel patches, and not at the

open spaces between the Blue mussel patches (Figure 2). The mussel abundance is given as mean abundance per m^2 ± standard deviation (SD).

Collector experiment

To get better knowledge about spatfall events, spat collectors of different substrate types (shells of Blue mussels, Pacific oysters, cockles or soft-shell clams) were offered in the field on mussel bed #12. The shells for the collectors for each shell type separately were stringed, with a hole in the middle of the shell, on three separate ropes that were 0.5 m in length. The three ropes were fixed as a bundle on the ground of the mussel bed with a distance of around 2 m between the different shell type collectors.

The collectors were installed on 26th April 2004 to ensure that they were on-site before the first settlement of oyster larvae. The first two of each type of collectors were collected on 30th September 2004 when no further oyster settlement was expected in the current season. Regularly visual controls were conducted approximately every two weeks, depending on weather conditions and the resulting accessibility of the mussel bed. The last collectors were retrieved on 10th August 2005, one year after settlement of the first oysters to identify the early individual growth. The size of all oysters settled on the collectors was measured for a length frequency analysis of the oysters. Spatfall events were identified by visual controls and the analysis of the length frequency. The growth in the first year was calculated from the difference in mean length of the oysters on the collectors. Further the number of Pacific oysters per cm^2 (length x width of the oyster shell) which settled on the first two collector was calculated.

Biomass production

Pacific oyster specimens, covering the size spectrum of the population, were collected on mussel bed #2, #3 and #12 in 2004 and 2005 for biomass determination. The tissue of each individual was totally removed from the shell, to determine the wet mass (WM), the dry mass (DM) and the ash free dry mass (AFDM) of the tissue. For the DM the tissue was dried at 80 °C over night to a constant weight. The tissue was incinerated at 450 °C for 6 hours to obtain the AFDM.

Annual production (*P*), mean annual biomass ($\overline{B}$), annual $P / \overline{B}$ ratio, mean body mass, and the individual production was calculated using the mass specific growth rate method, according to Brey (2001). The von Bertalanffy growth function (VBGF), the regression equation (relationship of biomass (WM) versus shell length) and the length - frequency - distribution was used for calculations with the mass specific growth rate method. The calculation with the WM gave the best regression equation for the relationship with shell length, for the conversion into DM and AFDM the conversion factors are given.

Data analysis

Abundance distributions of the Pacific oyster and the Blue mussel

Results are presented as arithmetic means ± standard deviation. For those data that were not normally distributed (tested with Kolmogorov-Smirnov-Test), like the Pacific oyster data, Wilcoxon signed-rank test or the Friedman test were used. For normally distributed data (Blue mussel data) we used a one-way ANOVA to test changes over the years. All tests were two-tailed with level of significance of $P \leq 0.05$.

Length frequency distribution and population growth

Length frequency distributions were determined for all investigated mussel beds (data not shown). The length frequency distribution data were fitted to the “von Bertalanffy growth function” (VBGF) with the program FiSAT II. For the calculation of the growth constant (*K*) of the VBGF, the Shepherd's method from the FiSAT II was used. The length L_∞ is given from FiSAT II and represents the largest accepted length present in the population. For statistical comparison of the mean growth constants (*K*) of the Pacific oyster populations and Blue mussel populations the T-test was used, test were two-tailed with level of significance of $P \leq 0.05$.

Results

Temperature measurement

The monthly mean temperature on the ground of the mussel bed varied between winter to summer on mussel bed #3 from 2.4 ± 1.7°C to 20.5 ± 2.6°C and on mussel bed #12 from

1.2 ± 1.3°C to 20.5 ± 3.0°C (Figure 3). In Figure 4 the diurnal temperature variation of one summer day with low tide during day is presented, together with the approximated tidal level. During the diurnal temperature course a peak of warm water is visible after the tide reached the mussel bed.

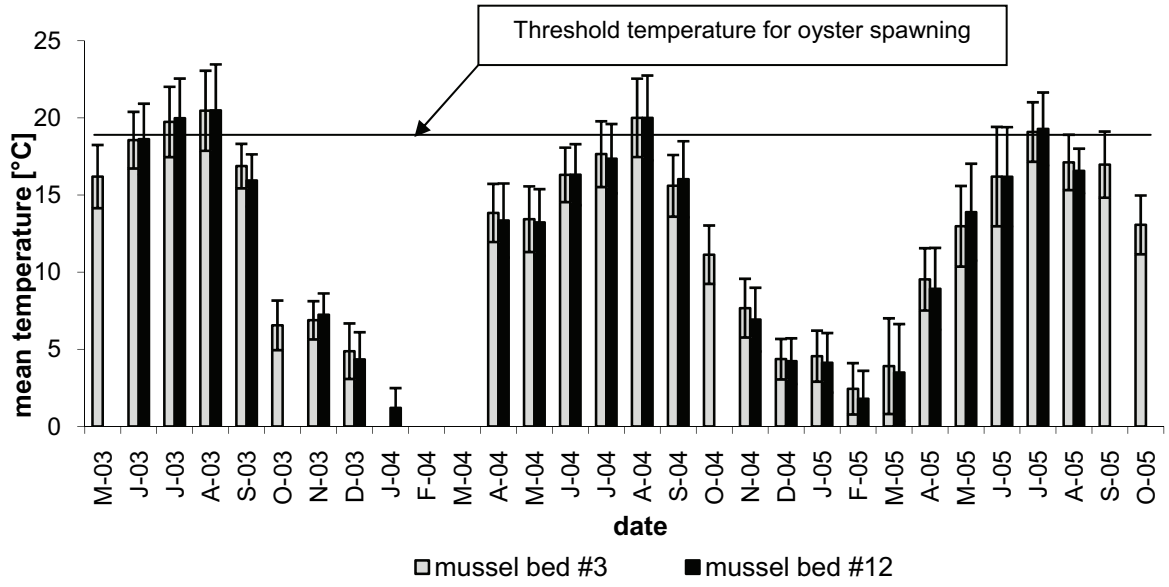

Figure 3: Mean temperature ± standard deviation on the ground of the mussel bed #3 and #12.

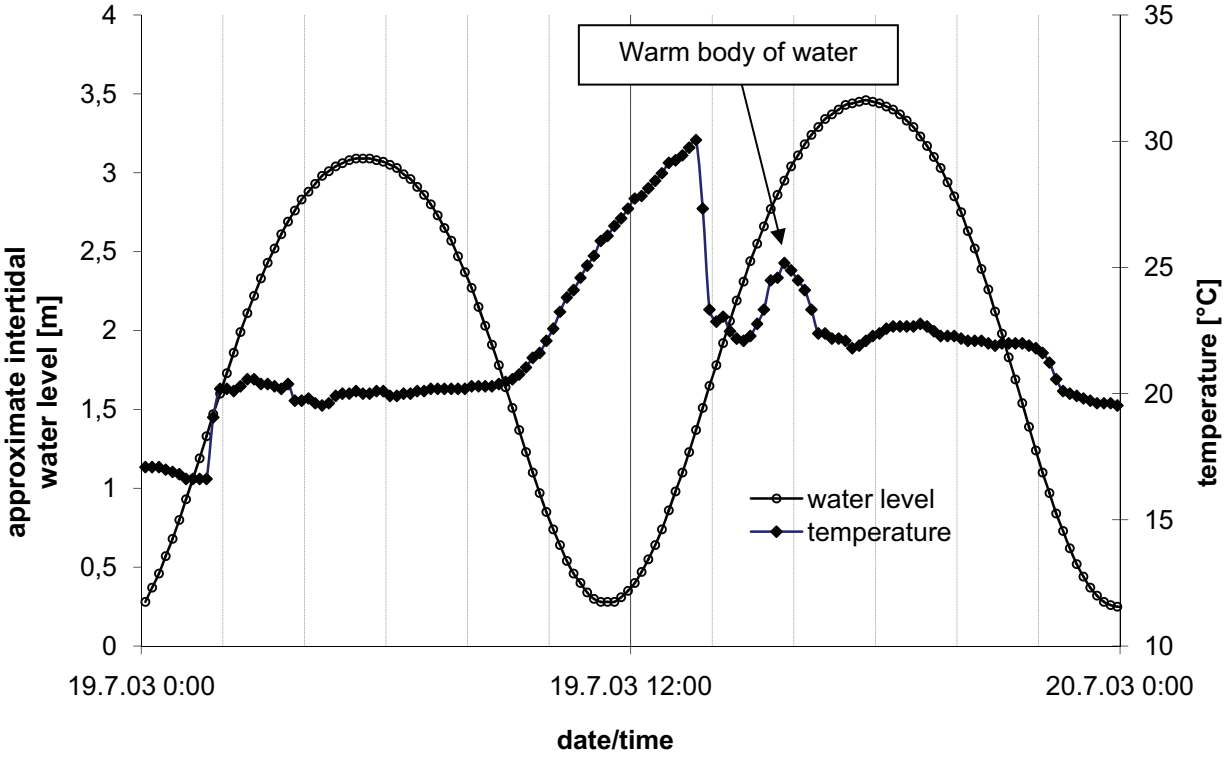

Figure 4: Diurnal temperature with low tide during the day.

Settlement of the Pacific oyster

The hard substrate covering the mussel beds in percent of the entire investigated mussel bed area of the 15 mussel beds was in 2003 43.5 ± 19.6%, in 2004 25.5 ± 17.0% and in 2005 17.0 ± 12.0%.

Substrate types on which we detected Pacific oysters were: cockle shells, live Blue mussels and Blue mussel shells, live and dead barnacles (different species), shells of Soft-shell clams (*Mya arenaria*), live and dead Common periwinkle (*Littorina littorea*) and live Pacific oysters and oyster shells. Live and dead substrate was not differentiated for further analysis, as the state of the substrate at the moment of settlement was unknown. The frequency of detected substrate (in percent) on which the oysters settled (Figure 5) changed in the course of the bio-invasion. In the first study year (2003), 40% of the oysters were found on

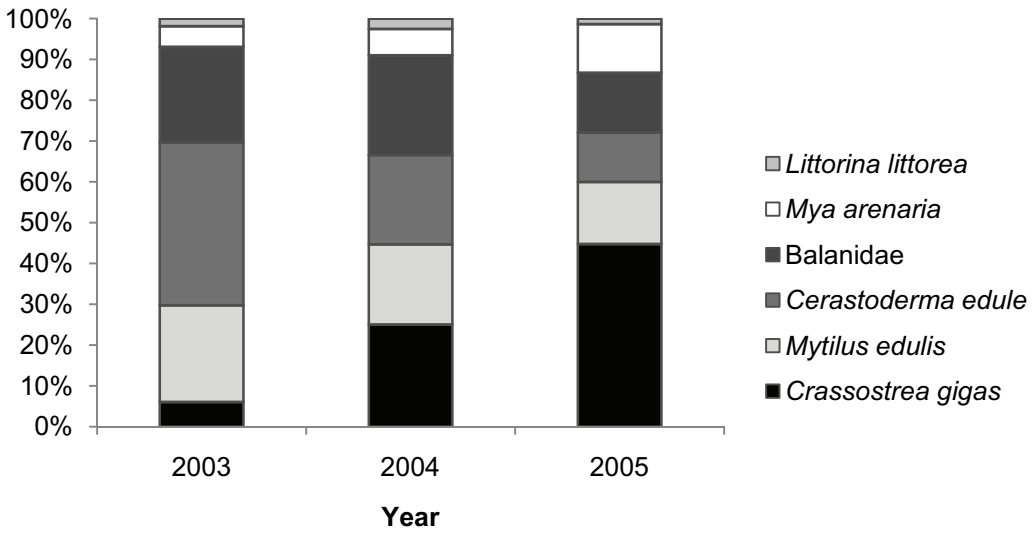

Figure 5: Substrate used for settlement by the Pacific oyster expressed as percentage of all counted oysters from all examined mussel beds (2003 N=7183, 2004 N=20381, 2005 N=38670).

cockle shells, 24 % on Blue mussels and 23% on barnacles. The use of the own species as settlement substrate increased from 6 % in the first year to 25 % in the second year and in the third year up to 45 %. In the second year (2004) cockle shells (22 %), Blue mussel (20 %) and barnacles (24 %) had an important part as settlement substrate. In the third year oysters were

found to 12 % each on cockles and soft-shell clams and to 15 % each on Blue mussels and barnacles. The settlement we found on periwinkles in the three years ranged from 1% to 3%.

The collector experiments showed six separate spatfall events between August and September 2004. Regularly visual controls showed that no Pacific oyster spat settled on the collectors after August 2004. On average most Pacific oyster settled on cockle shells (0.96 / cm^2), 0.46 / cm^2 on Pacific oyster shells, 0.37 / cm^2 on shells of the soft-shell clam and only 0.26 / cm^2 on Blue mussel shells.

Abundance comparison of Pacific oysters and Blue mussels

In comparison the Pacific oyster populations showed in total rather an increase, whereas the Blue mussel populations varied more between mussel beds.

The abundance of the Blue mussel (n = 12 per year and mussel bed, Figure 6a) showed a significant increase over the three years on one of nine mussel beds (mussel bed #15 p = 0.001), whereas the abundance decreased on four mussel beds (mussel bed #1 p = 0.000, #3 p = 0.017, #8 p = 0.003 and #10 p = 0.004). On the other mussel beds no significant change of the abundances were identified.

The abundance of the Pacific oyster (n = 100 per year and mussel bed) showed a significant increase on eight of the nine mussel beds (mussel bed #1 p = 0.000, #3 p = 0.000, #5 p = 0.005, #8 p = 0.000, #10 p= 0.004, #11 p = 0.000, #12 p = 0.000 and #14 p = 0.000), while no change occurred on mussel bed #15 (Figure 6b).

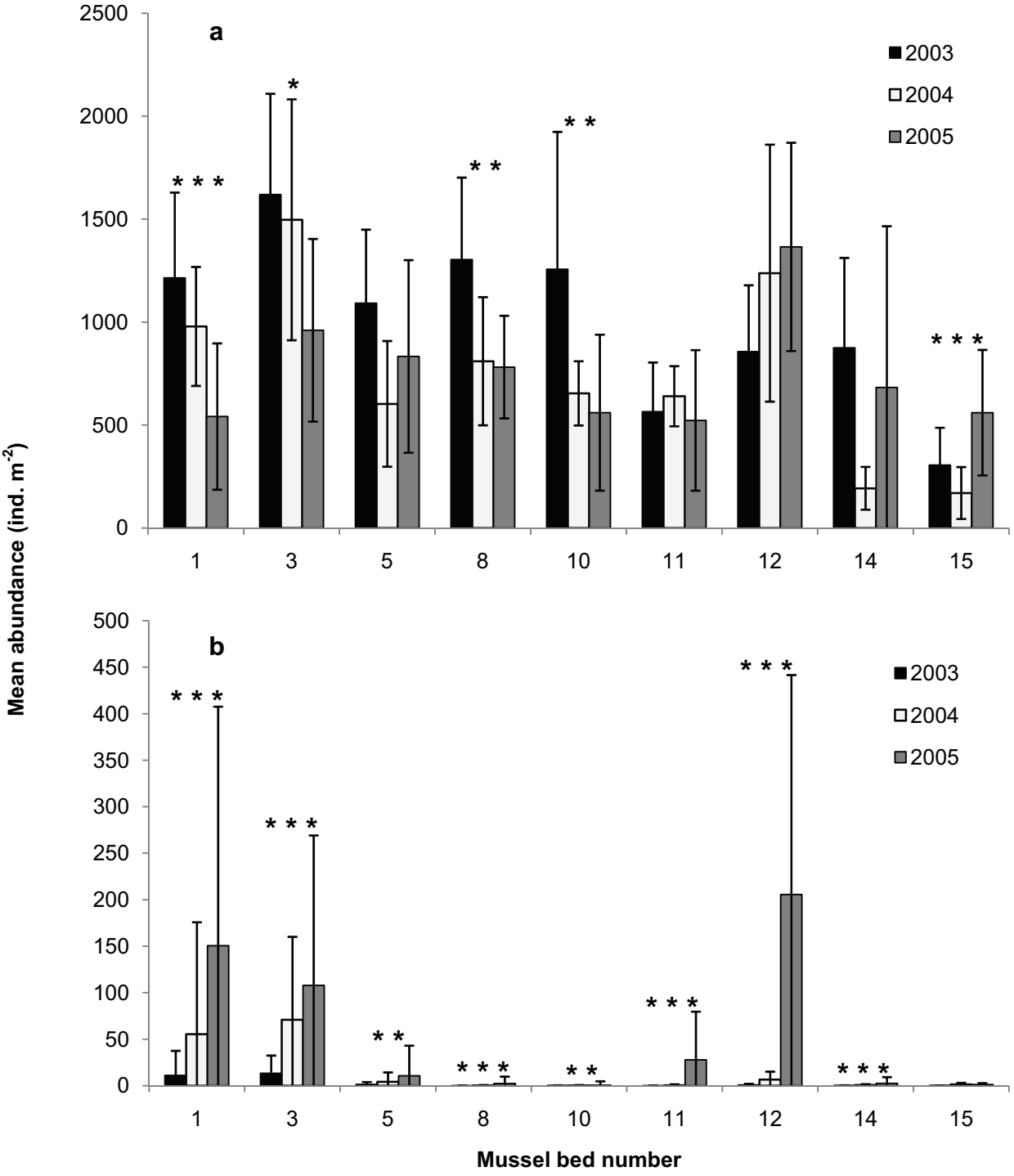

Figure 6: Mean abundance ± standard deviation of the Blue mussel (a) and the Pacific oyster (b) on the investigated mussel beds (see Figure 1). Significant changes over the three investigated year were tested for mussels with one-way ANOVA (n = 12) for oysters with Friedman-Test (n = 100); p values
* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.

Population growth comparison of the Pacific oyster and the Blue mussel

The mean growth rate of Pacific oyster and Blue mussel populations, comparison of the mean growth constant (*K*) (Table 1), from the stocks of mussel bed #1, #3, #11, #12 and #14,

showed no significant difference. The comparison, however, showed a higher mean growth constant for the Pacific oyster population. The highest growth rate for the Pacific oyster was found on mussel bed #11 with K = 0.990 y^{-1} and for the Blue mussel on mussel bed #3 with K = 0.650 y^{-1}. Only on mussel bed #3 the growth constant showed a twofold higher population growth of the Blue mussel compared with the Pacific oyster.

Table 1: L_∞ and growth constant (K) of Pacific Oyster populations calculated for the three years of investigation (Data published in Schmidt et al. 2008) and L_∞ and growth constant (K) of Blue mussel populations on the same mussel beds (locations see Figure 1). Statistic comparison of the growth constant (K), using the T-test, showing no significant difference.

	C. gigas		*M. edulis*	
Mussel Bed	L_∞ *(mm)*	$K\ y^{-1}$	L_∞ *(mm)*	$K\ y^{-1}$
# 1	207,90	0,440	80,85	0,320
# 3	186,90	0,300	71,40	0,650
# 11	129,15	0,990	80,85	0,340
# 12	139,65	0,580	74,55	0,310
# 14	92,40	0,670	80,85	0,130
Mean	151,20	0,596	77,70	0,350
SE	20,70	0,117	1,99	0,084

Individual growth during the first year after settlement

The individual growth during the first year was calculated from oysters that settled on the collector experiments carried out on mussel bed # 12. For the calculation the amount of individuals in 2004 was N = 4067 and in 2005 N = 716. In the first year the mean growth of the Pacific oyster was 32 mm y^{-1}. The predominant growth occurred in the ensuing summer after the spat fall that happened between August and September.

Biomass production

The mean annual Pacific oyster abundance from mussel bed # 2, # 3 and # 12 for the years 2004 and 2005 (Table 2) represented a mean annual biomass in a range of 9.8 g WM $m^{-2}y^{-1}$ to 924.9 g WM $m^{-2}y^{-1}$. Annual production ranged between 115.0 g WM $m^{-2}y^{-1}$ and 1140.2 g WM $m^{-2}y^{-1}$, and the annual $P / \overline{B}$ ratio ranged between 1.2 and 2.9. The mean body mass (WM) ranged between 1.392 g and 3.565 g. The individual production reaches its highest value at a certain length, it ranged between 13.812 g WM $m^{-2}y^{-1}$ at 88 mm to 3.138 g WM $m^{-2}y^{-1}$ at 58 mm.

Table 2: Biomass production of Pacific oyster populations from three selected mussel beds (#2, #3, #12 see Figure 1). Conversion factors were estimated for the conversion of g WM into g DM and g AFDM. The regression equation was calculated as ratio between shell length and biomass (WM).

Mussel Bed, year	**# 2, 2004**	**# 2, 2005**	**# 3, 2004**	**# 3, 2005**	**# 12, 2004**	**# 12, 2005**
Mean annual biomass (B = g WM $m^{-2}y^{-1}$)	483.2	924.9	134.2	224.2	9.8	286.2
Annual production (P = g WM $m^{-2}y^{-1}$)	839.6	1140.2	261.3	258.8	115.0	831.1
Annual *P/B* ratio	1.7	1.2	1.9	1.2	2.9	2.9
Mean body mass (M in g WM)	3.6	3.1	1.9	2.1	1.5	1.4
Individual production (g WM $m^{-2}y^{-1}$) at length	10.4 g at 93 mm	6.1 g at 78 mm	7.1 g at 88 mm	3.1 g at 58 mm	13.8 g at 88 mm	9.9 at 83 mm
Conversion factor WM to DM	0.181	0.2	0.183	0.147	0.233	0.225
Conversion factor WM to AFDM	0.133	0.151	0.15	0.107	0.197	0.166
Regression equation (g WM versus length[mm])	$y = 0.0024\ x^{1.9}$	$y = 0.0043\ x^{1.68}$	$y = 0.002\ x^{1.92}$	$y = 0.0097\ x^{1.44}$	$y = 0.000061\ x^{2.77}$	$y = 0.0003\ x^{2.37}$
r^2	0.82	0.787	0.848	0.739	0.912	0.874
n	31	59	61	55	17	45

Discussion

This study was conducted to indicate a potential impact of the invasive Pacific oyster on the native Blue mussel and to investigate if environmental conditions (temperature, salinity and settlement substrate) which are essential for the growth and reproduction of the Pacific oyster were available in the soft sediment tidal flats of the Wadden Sea. Beside shell fields, harbours and dikes, Blue mussel beds are the only further hard substrate for the settlement of the Pacific oyster in this ecosystem, which brings the Pacific oyster in a spatial proximity to the Blue mussel. Therefore our investigation was concentrated on Blue mussel beds along the East Frisian Wadden Sea coast. We investigated the impact of the oyster on the Blue mussel population and compared the population dynamic of both species. Furthermore, we investigated the biomass production of oyster, which could be used by other species as food and can estimate a further influence on the food web of the ecosystem. The biomass data of the oyster were compared with literature data of the Blue mussel.

Abiotic factors

Temperature and salinity are basic abiotic factors for reproduction and growth of oysters (Ayres 1991, Cardoso et al. 2007, Chávez-Villalba et al. 2002, Diederich et al. 2005, Eno 1994, Kenny et al. 1990, Ortega and Sutherland 1992, Quayle 1988, Ruiz et al. 1992, Spencer et al. 1994). Therefore, in habitats exposed to tides, it is necessary to know not only the water temperature, but also the temperature to which the oysters were exposed during low tide.

Table 3: Temperatures known as important for the development of the Pacific oyster.

Temperature	Comment	Reference
~ 10 °C	Gonad development, which begins in May in the North Sea.	Neudecker 1985
~ 18 - 19.5 °C	Temperature for spawning, which is initiated by temperature, chemical stimulation or a combination of both.	Mann 1979, Quayle 1969, Ruesink et al. 2005, Spencer et al. 1994
72 h at 14 °C 28 h at 22 °C	Temperature for egg development and time to reach the veliger stage, at two different temperatures	Loosanoff & Davis 1963
≥ 20 °C	Temperature for larval development, the water temperature must be for at least three weeks at the optimal value for a near optimal growth, lower water temperatures increase the time of the free-swimming period	Magoon & Vining 1981, Quayle 1988, Kennedy & Breisch 1981

Various authors described temperatures necessary for the oyster development (Table 3). As temperatures for the oyster development were reached during each study year, temperature could not be the limiting factor for the growth and propagation of the Pacific oyster. Furthermore the warm water peak during flood tides could work as stimulation necessary for a synchronous initiation of spawning, which secures a successful fertilisation of the Pacific oyster (Hidu and Haskin 1971, Ingle 1951, Loosanoff and Engle 1940, Lutz et al. 1970). Salinity was within the optimum range for oysters of 25 - 35 ‰ (Quayle 1969) in the entire study area. Thus, the invasive Pacific oysters encountered favorable environmental conditions during the study years (2003 - 2005), which was also reflected by the spat falls detected on the collectors, their fast growth and the overall increase of the oyster population in the Wadden Sea (Diederich et al. 2005, Schmidt et al. 2008). The individual growth rate of the oyster is very similar in the entire Wadden Sea during their first three years, approximately 40 ± 15 mm^{-y} (for comparison see Diederich 2006, Schmidt et al. 2008). Our examination showed that growth takes place during the summer months when plankton blooms occur in the Wadden Sea (Joint and Pomroy 1993). Seasonal variations in the Pacific oyster condition depending on food availability (Kang et al. 2000). The most important factor for Pacific oyster development, growth and reproduction is certainly the temperature. Diederich et al. (2005) described that the oyster recruitment only took place during high late summer water temperatures, considering the temperatures and oyster spat falls of the last 17 years. The high

water temperatures during our study were part of a warm period, which may be caused by climate change (Loewe et al. 2006). The influence of water temperature is furthermore shown by change in recruitment of other bivalves (*Cerastoderma edule*, *Macoma balthica*, *Mya arenaria*, *Mytilus edulis*) from the North Sea, which was also discussed as influenced by climate change (Philippart et al. 2003). Alterations in communities occurred in the past accompanying alterations between glacial and non glacial periods (see Graham et al. 2003) but also changing conditions in the recent past lead to alterations of the benthos community of the North Sea (Kröncke et al. 1998). Temperature seems to be one of or the mayor factors which influences also native populations (see Pörtner and Knust 2007). If a non-indigenous species is better adapted to the changed environment it can prevail over native species, which could happened during the process of community change because of climate change (see Nehring 2003, Stachowicz et al. 2002, Walther et al. 2002). For this reason the Pacific oyster will benefit from climatic changes and may get well established in the Wadden Sea ecosystem.

Important for the spread of Pacific oyster in the tidal flats are not only factors like temperature, but also the availability of food and suitable settlement substrate. The latter is limited in the Wadden Sea. The most abundant hard substrate in the East Frisian Wadden Sea were Blue mussel beds where the initial settlement of the Pacific oyster took place, described by various authors (see Drinkwaard 1999, Reise 1998, Schmidt et al. 2008, Wehrmann et al. 2000). Yet, a colonisation of artificial substrates was also reported (Tydeman 1999). Our investigations showed that the oysters used mostly cockle shells (*C. edule*) at the beginning of the invasion, which have surface irregularities similar to oyster shells (Crisp 1967; Galtsoff 1964). With the increasing abundance of oysters, the settlement substrate shifted to the use of conspecifics. This shift can be explained by an alluring pheromone which is exuded from the oyster shell (Arakawa 1990). The use of conspecifics as settlement substrates gives the oyster an opportunity for a further spread, especially in environments where other adequate hard substrate is limited. In our study, available hard substrate was reducing on the investigated mussel beds, which could be due to the general decrease of Blue mussels (see

below). Mussels deliver a structure in which shell material is hold by byssus threads, without this structure shell material can be washed away by waves and currents.

Competition or Co-existence of the Pacific oyster and the Blue mussel

The spread of the invasive Pacific oyster on Blue mussel beds can lead to competition for space and a possible displacement of Blue mussels. This possibility has been documented in the case of the introduced mussel *Mytilus galloprovincialis* outcompeting the native *Aulacomya ater* on the west coast of South Africa (Van Erkom Schurink and Griffiths 1990).

Our comparison of the abundances of Blue mussels and Pacific oysters showed no consistent pattern between abundance variations of Blue mussels with increasing abundances of oysters. The decrease of the Blue mussel stock can be due to its general decrease (see Figure 7) since the mid 1980s (Herlyn 1996, Herlyn and Millat 2000, Michaelis et al. 1995, Obert and Michaelis 1991, Zens et al. 1997) which was only interrupted temporarily by the extraordinary spatfall from summer 1996.

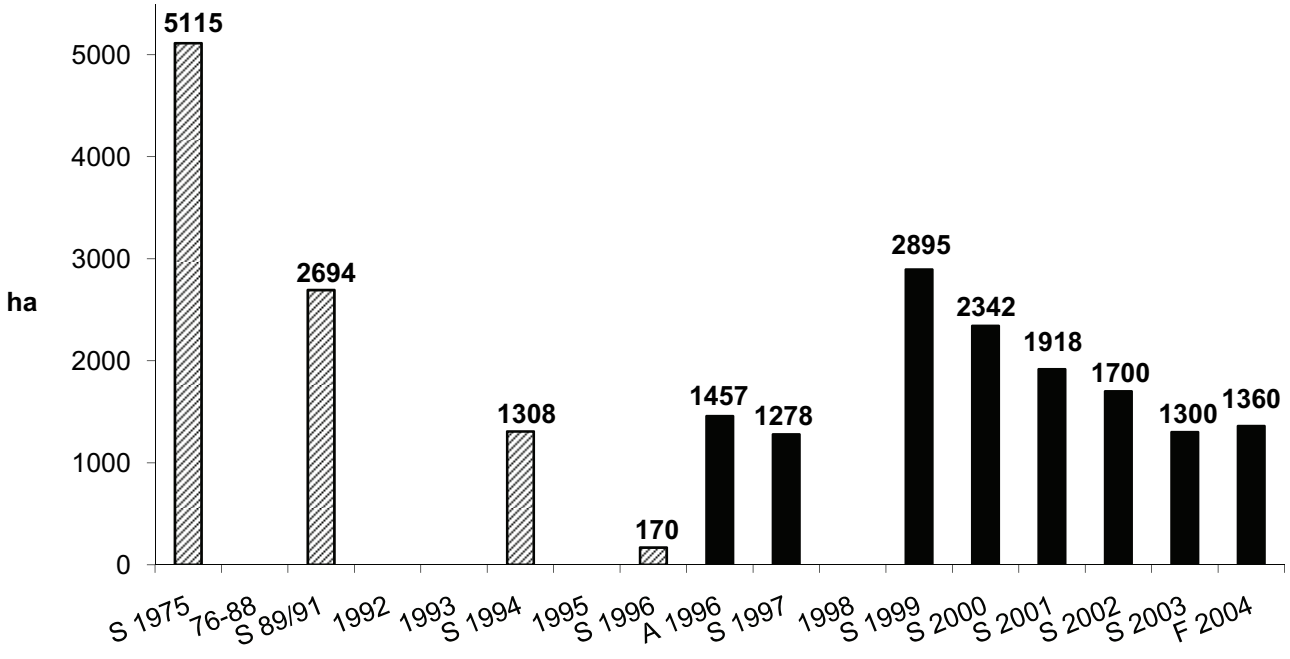

Figure 7: Area covered by Blue mussel beds on the East Frisian Wadden Sea tidal flats (sampling time: S = summer, A = autumn, F = spring). The black columns marked the years after the large spat fall in 1996. Figure changed after (Herlyn and Millat 2004)

Although there was no significant difference between the calculated growth constant (*K*) of both species, on most mussel beds the growth constant of the oyster was higher than that of mussels. However, on mussel bed #3 mussel population growth was twofold higher than oyster growth. Thus, Blue mussels may not be outcompeted by the Pacific oyster in the Wadden Sea. Even though the environmental condition, such as temperature, are very similar in the entire Wadden Sea it is very likely that the abundances of both species are different in the different areas of the Wadden Sea due to the differences in population growth of the oyster between the investigated mussel beds (see Schmidt et al. 2008). But further it is likely that the oyster, where the population is established, may have a positive effect on the population of the Blue mussel because of the oyster reef structure which could give shelter to the Blue mussel. Frandsen and Dolmer (2002) described that the complexity of the substrate increased the Blue mussel survival significantly, due to a decrease in predation pressure. The substrate structure created by the oysters may provide habitat that supplies a refuge for many species in the Wadden Sea. The structure of the mussel beds will change to mixed beds with the Pacific oyster as habitat engineer. A likely coexistence of both species was also discussed for the northern part of the Wadden Sea (Diederich 2005a, Diederich et al. 2005, Nehls et al. 2006). That the Pacific oyster will influence the Wadden Sea ecosystem, especially on the structure of the mussel bed, is also shown by the high biomass production of the Pacific oyster in comparison to the Blue mussel. Munch-Petersen and Kristensen (2001) gave an average $P / \overline{B}$ ratio of 0.4–0.5 for Blue mussel populations in the Danish Wadden Sea. The $P / \overline{B}$ ratio we calculated for the Pacific oyster is in the range of 1.2 to 2.9, so the production of the oyster is around fivefold higher. Through its fast growth the oyster accumulates a high amount of biomass in a short time, which will increasingly influence the ecosystem and the food web of the Wadden Sea ecosystem. The use of the oyster by higher consumer levels as prey is negligible so far, because only low predation pressure is expected for oysters, e.g. by benthic predators (Diederich 2005b) and by seabirds (Cadee 2001, Scheiffarth et al. 2007).

Conclusion

This study shows that the Wadden Sea is a suitable habitat for the growth of the Pacific oyster and enables the oyster to establish self sustaining populations. In consideration of ongoing climate change the oyster apparently benefited from higher temperatures in the Wadden Sea, which are necessary for a successful recruitment (see Diederich et al. 2005).

In the initial phase of the invasion mostly Blue mussel beds provided the substrate for settlement and a further spread. Once the oyster started to build up a population they provide their own settlement substrate and therefore have the ability to enlarge their population. This results in a very successful invasion of this hard substrate inhabitant in the soft sediment environment of the Wadden Sea. The spread of the Pacific oyster on mussel beds may lead to competition between the oyster and the mussel, but may not outcompete the Blue mussels. The high biomass production by the Pacific oyster lets up anticipate functional changes in the Wadden Sea ecosystem.

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Chapter 3

Spatial overlap and feeding competition between an introduced and an indigenous epibenthic bivalve on tidal flats in the southern North Sea

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Schmidt A, May P, Wehrmann A, Dittmann S (in prep. for the Journal of Sea Research) Spatial overlap and feeding competition between an introduced and an indigenous epibenthic bivalve on tidal flats in the southern North Sea

Abstract

Competition following an invasion can influence the recipient community when invasive species have advantages over indigenous species by faster growth or higher reproductive success. Here, we investigate spatial overlap and food competition between the introduced Pacific oyster *Crassostrea gigas* and the indigenous Blue mussel *Mytilus edulis* in the Wadden Sea of the southern North Sea, where the Pacific oyster was introduced about 20 years ago. Oysters and Blue mussels are the only epibenthic bivalves in this soft-sediment intertidal environment. Under the hypothesis that the two species compete for space on a small scale, we estimated spatial overlap by analysing the density of oysters and mussels. Our data showed that Pacific oysters settled in areas which were formerly used by Blue mussels. However, a positive correlation of oyster and mussel densities indicated a coexistence of both species. This co-occurrence could lead to food competition in densely populated oyster / mussel beds. To estimate the competition for food we analysed the filtration rates of both species, which was higher for Blue mussels than for Pacific oysters, indicating an advantage in filtration efficiency for the mussel. This filtration benefit explains the ability of the smaller mussel to live deep inside the structure of the larger oysters, which was the case on many mixed mussel beds. Considering the present growth of the oyster population it is possible that all former mussel beds of the tidal flats of the southern North Sea will be replaced by mixed mussel / oyster beds in the future.

Keywords

Bio-invasion, Wadden Sea, Pacific oyster, Blue mussel, non-indigenous species, competition

Introduction

Invasive species can either invade into free ecological niches of an ecosystem or outcompete native species. In both cases, invasive species have an impact on the native community of the affected ecosystem (Carlton 2002, Crooks & Khim 1999, Mack et al. 2000), which can affect species interactions, the food-web or ecosystem processes (Grosholz 2002).

Often the invasive species compete directly with native species for resources such as food or space (Buss 1979, Kotta & Olafsson 2003, Neidemann et al. 2003). That competition for space and food can be interdependent was shown for two bryozoan species *Onychocella alula* and *Antropora tincta* (Buss 1979). The interdependency of competition (here intra-specific competition) for space and food was also tested on Blue mussels *Mytilus edulis*, which were crowded into a very confined space and revealed a reduced filtration rate of small individuals due to a reduced shell opening caused by the limitation of space (Frechette et al. 1992).

Competition between a native and an invasive species can ultimately result in the replacement of the native species. The mud snail *Batillaria attramentaria*, introduced to the Pacific coast of North America together with the imported Pacific oyster *Crasostrea gigas*, has replaced their native ecological equivalent, the California Horn Snail *Cerithidea californica* (Byers 1999). Another example is the introduced mussel *Mytilus galloprovincialis*, which has outcompeted the indigenous *Aulacomya ater* as the dominant mussel on the west coast of South Africa (Van Erkom Schurink & Griffiths 1990).

Competition for space is common in rocky shores (Airoldi 2000, Barnes & Rothery 1996, Connell 1961, Paine 1984, Sebens 1986), however, it is not regarded as important in structuring soft-sediment benthos (Peterson 1979, Peterson 1991). Organisms living in soft sediments occupy a three-dimensional space (on the surface and in the sediment), which minimises opportunities for direct competition for space as species can evade this competition by living at different depths within sediments (Peterson 1979), which was shown by experimental manipulation of bivalve densities (Peterson 1977).

Before the bio-invasion of the Pacific oyster into the Waddden Sea, other invaded bivalves like the endobenthic soft-shelled clam *Mya arenaria* and the Razor clam *Ensis americanus* found free ecological niches, with no negative influence on the ecological community (Reise et al. 2002, Reise et al. 2005).

Competition for food could be the more important process than competition for space, controlling standing stocks and community composition of benthic macrofauna at a variety of spatial and temporal scales (Peterson & Black 1987, Lenihan & Micheli 2001). Experimental manipulations showed that the basket shell *Corbula gibba*, which was introduced to Port Phillip Bay (Victoria, Australia), had a significant effect on the size and growth of juvenile Commercial Scallop *Pecten fumatus* and the mechanism was suggested to be competition for food (Talman & Keough 2001).

In this study we investigated the competition for space and food of two bivalves living in a soft sediment environment, the indigenous Blue mussel *Mytilus edulis* and the non indigenous Pacific oyster *Crassostrea gigas*.

The Pacific oyster was first introduced in 1964 to the southern North Sea for Aquaculture at the Oosterschelde (Drinkwaard 1999), from where it spread first into the Wadden Sea of the Netherlands and subsequently into the East Frisian Wadden Sea, Germany (Bruins 1983, Dankers et al. 2004, Schmidt et al. 2008, Wehrmann et al. 2000). For the Pacific oyster, Blue mussel beds are the most frequently available natural hard substrates at the East Frisian tidal flats. Consequently, the Pacific oyster invades into space that is already used by the Blue mussel. This spatial proximity brings the Blue mussel and the Pacific oyster into a potential competition for space and food as both species use the limited hard substrate and the same food source. Therefore, we want to address the question whether there is a competition for these resources between the oyster and mussel or not. As an indicator for space competition we used the population densities of oysters and mussels on a mussel bed. To evaluate the possibility of an existing competition for food, we analysed filtration rates of both species.

Material & Methods

Study site

Investigations were carried out on a mussel bed in the western part of the East Frisian Wadden Sea (Germany), south of the island of Juist (53°38.49'N 6°56.56'E; Figure 1). The mussel bed is known as constant in shape (Millat pers. com.) and covered an area of approximately 37 hectare (measured in 2003). The Wadden Sea in that area is characterised by muddy to sandy tidal flats with a semidiurnal tide cycle and a tidal range of 2.5 m. To assess the spatial extent of the mussel bed, all areas occupied by mussels not more than 25 m apart were mapped using a global-positioning system (GPS). This procedure follows the annual Blue mussel monitoring carried out by the Lower Saxony National Park administration (see also Herlyn 2005).

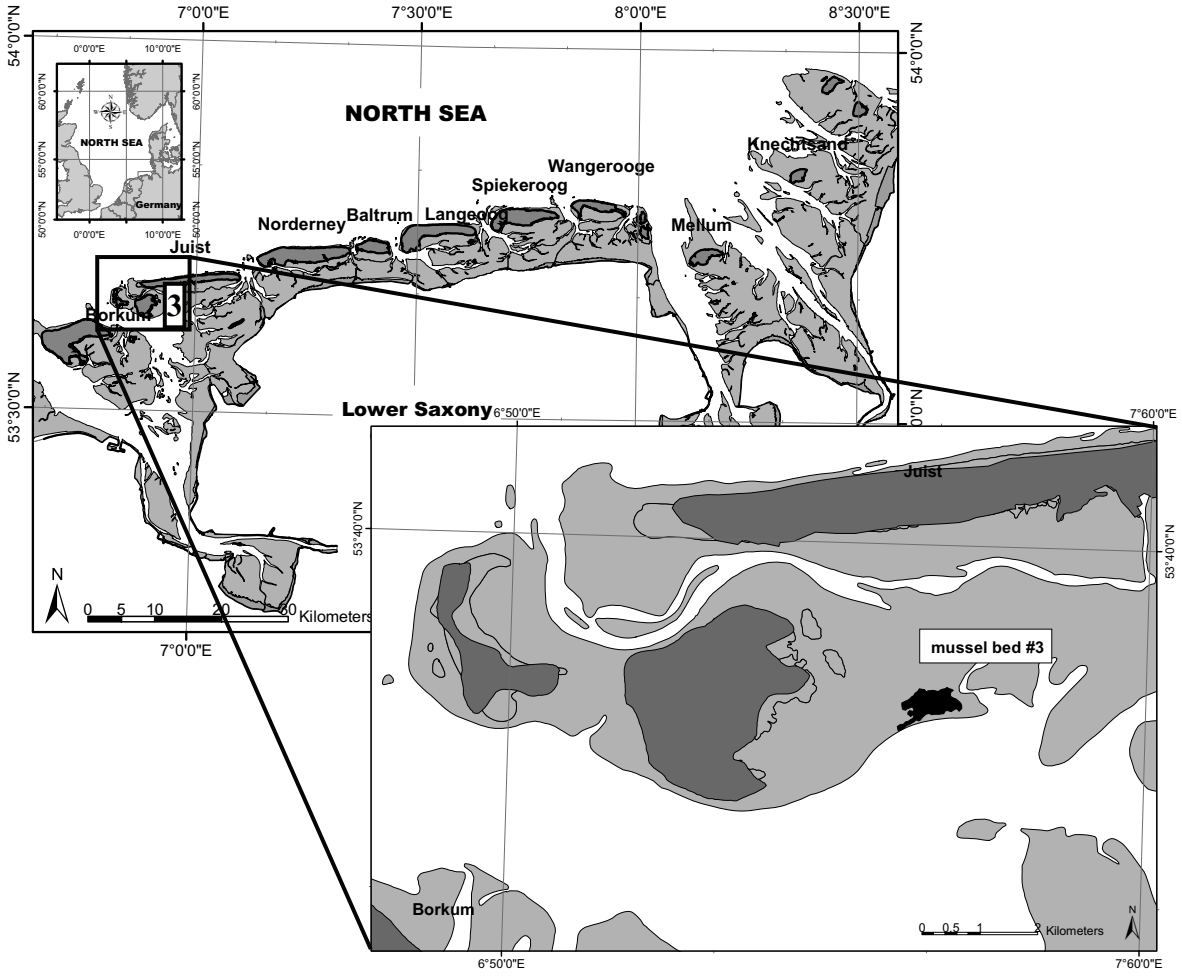

Figure 1: Detailed map of shape and location of the investigated mussel bed on the tidal flats of the Lower Saxony Wadden Sea, Germany. Dark grey areas indicate islands, light grey areas indicate tidal flats, and the area of the mussel bed is indicated in black.

Pacific oyster and Blue mussel survey

The determination of Pacific oyster and Blue mussel presence and abundances was carried out in spring 2003, 2004 and 2005, before the spat fall of the respective year took place. Over the whole mussel bed area, 100 sampling sites were located by using random number generated coordinates. For exact positioning in the following years, the sites were marked with bamboo sticks. At each sampling site, Pacific oyster and Blue mussel densities were determined in the field within a 1 m^2 quadrate, and the counted specimens were left at the sampling sites. The Pacific oysters were counted (individuals m^{-2}) and the cover of living Blue mussels (percent m^{-2}) was estimated in five percent steps over the quadrate. Coverage above zero and below five percent was defined as one percent. From 2004 onwards the quadrate size for the Pacific oyster counting was reduced to 0.25 m^2 because of their high abundance.

For a better description of the occurrence of Blue mussels in between the Pacific oyster, we compared the abundance (individuals m^{-2}) of oysters and mussels in summer 2005. Samples were taken from three different sites of the mussel bed and chosen on the basis of the Pacific oyster abundance in the year 2004. The first site (C) had the highest oyster abundance, the second site (M) had the lowest oyster abundance and the third site (C/M) had an oyster abundance in between site C and M. On each site, samples (n = 5) were taken with a core of 177 cm^2 (15 cm diameter). Samples were taken to the laboratory, where all oysters and mussels were counted.

Filtration experiments

Oyster and mussel specimens for the filtration experiments were collected in February 2005. Three age dependent size classes (covering the so far occurring total age span found in the field) were used: size class one were specimens aged between one and two years, size class two aged three to four years and size class three aged older than four years (based on data of Schmidt et al. 2008, Ahrendt & Bayerl 2003 and Bayne 1976). The specimens were cleaned from all periphyton and acclimatised for 2 – 3 weeks under constant temperature conditions of 15°C and a 12 hours dark and light cycle.

For the experiments, two algae species were selected which covered the size spectrum where oysters and mussels showed a high retention efficiency (*Isochrysis* spp. 4-8 µm and *Phaeodactylum tricornutum* 12-23 µm) (see Møhlenberg & Riisgård 1978, Ropert & Goulletquer 2000). The clearance rate was determined in a static system (see Riisgård 2001, Walne 1972). For acclimatisation to the experimental setup one specimen per setup was put into a 2 l beaker for 2 – 3 hours with cleaned seawater which was aerated. The experiment was started with addition of the alga suspension and stopped after three hours. For *Isochrysis* a starting concentration of 5.2 to 9.5 10^6 cells ml^{-1} and for *P. tricornutum* 1.8 to 2.7 10^6 cells ml^{-1} was used. Algal cell concentrations were determined by photometry at which the scattering of white light was measured (PERKIN-ELMER λ2).

The clearance rate was calculated using the following formula:

$$CR = \frac{V}{n} \times \frac{(\ln C_1 - \ln C_2)}{T}$$

(*CR* = clearance rate ($l\ h^{-1}\ ind.^{-1}$), *V* = volume of the beaker (l), n = number of specimens, C_1 = number of cells at the beginning, C_2 = number of cells at the end and *T* = duration of the experiment (h))

Data analysis

Pacific oyster and Blue mussel survey

An ordinary kriging (see e.g. Kappas 2001) was carried out with the abundance data of the Pacific oyster (individuals m^{-2}) and the cover of the Blue mussel (percent m^{-2}) and shown as prediction map (computation was done with the geostatistical analyst extension of ArcGIS 8.3).

Spatial distribution patterns were described through the variance / mean ratio (Random $s^2 = x$; Regular, uniform $s^2 < x$; Clumped $s^2 > x$) of the Pacific oyster (individuals m^{-2}) and the cover of the Blue mussel (percent m^{-2}) data.

Regression analysis was done between abundance data of the Pacific oyster (individuals m^{-2}) and the three investigated years, and the cover of the Blue mussel (percent m^{-2}) and the three years. In a further analysis oyster data were correlated (Spearman correlation) with

mussel data for each year separately. To test whether the two regression-coefficients b_1 and b_2 were significantly different from each other, t- distributed test statistics were computed:

$$t = \frac{|b_1 - b_2|}{\sqrt{\frac{s^2_{y1.x1} \cdot (n_1 - 2) + s^2_{y2.x2} \cdot (n_{2-2})}{n_1 + n_2 - 4} \cdot \left(\frac{1}{Q_{x1}} + \frac{1}{Q_{x2}}\right)}}$$

(b= regression coefficient; $s_{y.x}$=standard error of the mean; n = sample size; Q_x = denominator in the formula for the regression coefficient)

For the additional investigation in 2005 the oyster and mussel data were correlated with Spearman correlation for each sampling site separately.

Clearance rate

Data of the calculated clearance rate were tested for normal distribution using the Kolmogorov-Smirnov-Test with Lilliefors correction. When the data were not normally distributed, as was the case for the comparison between the oyster and the mussel data, non-parametric statistics (Mann-Whitney test) were used. When data were normally distributed the t-test was used, this was done to test differences between bivalve size classes or between the specimens within a class. Effects were considered to be statistically significant if the p-value was ≤0.05.

All analysis were carried out using the statistic software SPSS from SPSS Inc.

Results

Spatial competition

Between 2003 and 2005 Pacific oyster numbers increased where Blue mussel density had been the highest before. For both species (oyster and mussel) the spatial distribution pattern calculated with the variance / mean ratio (Table 1) showed a clumped distribution in all investigated years.

Table 1: Variance / mean ratio data of the Pacific oyster (individuals m^{-2}) and the cover of the Blue mussel (percent m^{-2}) (each year and species n = 100).

Species		Pacific oyster			Blue mussel	
Year	2003	2004	2005	2003	2004	2005
Mean	13.32	70.92	107.97	40.02	13.49	5.63
Variance	368.28	7958.86	25977.87	1231.74	399.81	125.49
variance/ mean ratio	27.65	112.22	240.60	30.78	29.64	22.29

The distribution calculated by kriging (Figure 2) showed in 2003, that oyster abundance was highest in the southwest of the mussel bed, whereas Blue mussels had a high abundance in most parts of the mussel bed. In 2004, the oyster showed a more equal distribution whereas the Blue mussel decreased at the edges of the mussel bed. By 2005, the oyster numbers had increased where the Blue mussel showed the highest abundance in the year before. The Blue mussel coverage decreased in 2005 in comparison to 2004 and showed a low density on the entire mussel bed.

Oyster density increased with years (linear regression: $y = -30.58 + 47.33\ x$, $r^2 = 0.12$, $F_{1, 298} = 39.223$, $p < 0.001$), whereas mussel density decreased with years (linear regression: $y = -17.2\ x + 54.1$, $r^2 = 0.25$, $F_{1, 298} = 98.032$, $p < 0.001$). The regression coefficient between oyster and years was significantly different to the coefficient between mussel and years (t-distributed test statistics: $t = 8.321$, $p < 0.001$)

Oyster and mussel densities were positively correlated in each investigated year (2003: $R_S = 0.393$, n = 100, $p < 0.001$; 2004: $R_S = 0.563$, n = 100, $p < 0.001$; 2005: $R_S = 0.667$, n = 100, $p < 0.001$; Figure 3).

The additional investigation in 2005 showed a positive correlation between Pacific oyster and Blue mussel abundances on site C ($R_S = 0.205$, n = 5, $p = 0.370$) with a mean oyster abundance of 456.9 ± 226.2 ind. m^{-2} and a mean mussel abundance of 576.5 ± 320.9 ind. m^{-2}, and on site C/M ($R_S = 0.051$, n = 5, $p = 0.467$) with a mean oyster abundance of 304.6 ± 98.8 ind. m^{-2} and a mean mussel abundance of 1011.7 ± 294.4 ind. m^{-2}, but a

negative correlation on site M (R_S = -0.115, n = 5, p = 0.427) with a mean oyster abundance of 87.0 ± 48.6 ind. m^{-2} and a mean mussel abundance of 1435.9 ± 467.2 ind. m^{-2}.

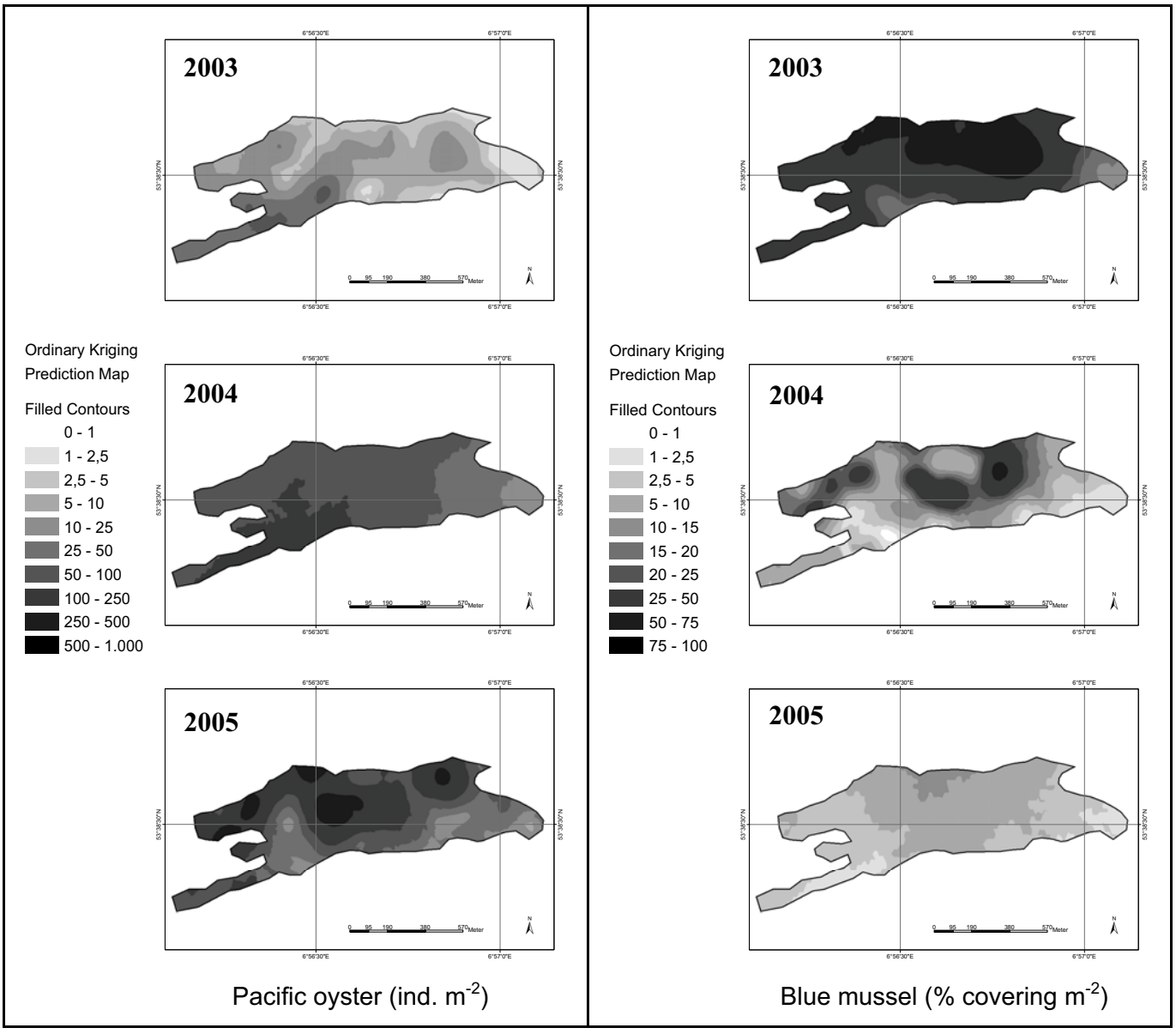

Figure 2: Spatial distribution of the Pacific oyster (filled contours showing graded areas from 0 to 1000 oyster individuals m^{-2}) and the Blue mussel (filled contours showing graded areas from 0 to 100 % covering m^{-2} by the mussels) on a mussel bed in the western part of the East Frisian Wadden Sea from 2003 to 2005.

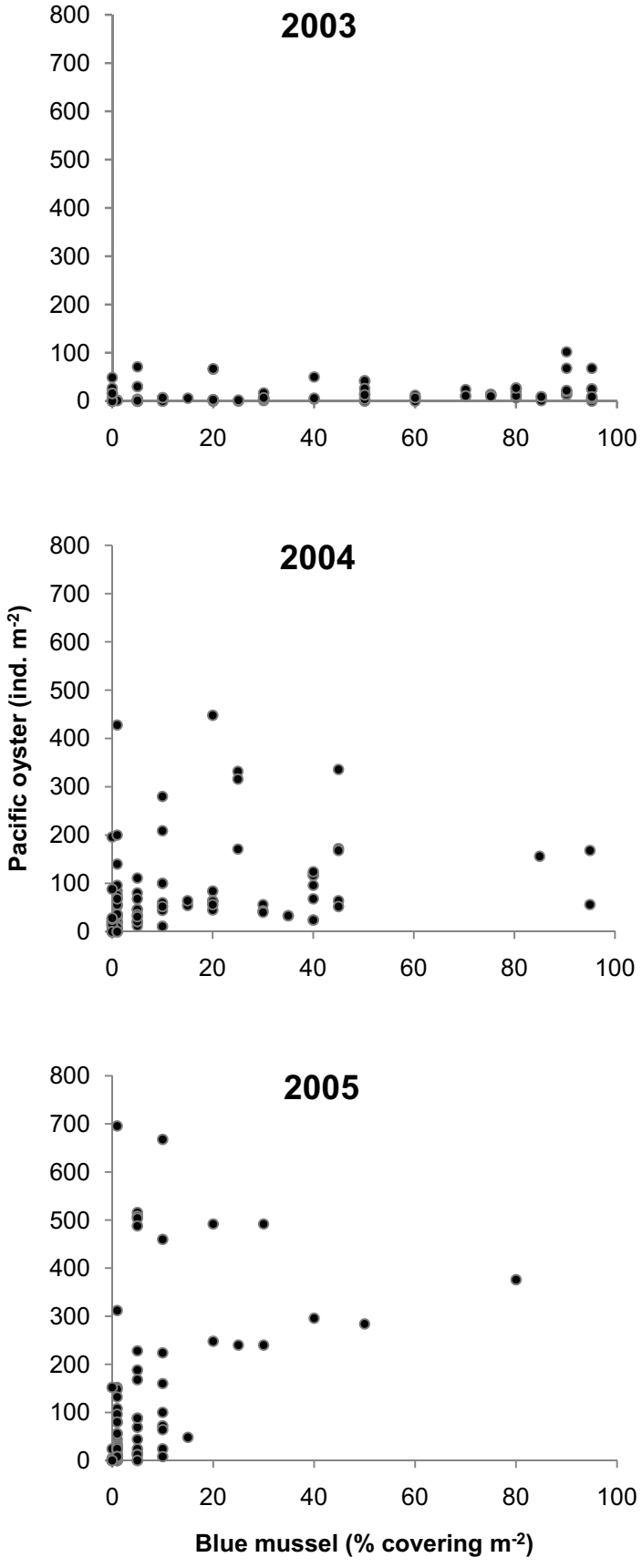

Figure 3: Correlation between Blue mussel and Pacific oyster densities.

Food competition

The mean clearance rates were given as litres per hour per ash free dry mass (L h^{-1}g $AFTM^{-1}$) and standard deviation, as an overview to see the differences between the bivalve species, the algal species and the size classes and all significant differences which were found are shown in Table 2. The mean clearance rate of Blue mussels was significantly higher ($p < 0.05$) than the clearance rate of Pacific oysters, when all experiments were combined for each bivalve species separately and the algae species was disregarded (A and B in Table 2, Figure 4). Clearance rates showed no significant differences between bivalve size classes or between the specimens within a class (Figure 5) when the algae species were disregarded.

Table 2: Mean clearance rates (litre per hour per ash free dry mass L h^{-1}g$AFTM^{-1}$) and standard deviation for oyster and mussel of different size classes, fed different diets of algae. Significant differences (U-tests) are described using the following notations, parenthesis indicating data grouped for calculations: A compared with B ($p = 0.04$); grouped CE compared with grouped DF ($p < 0.001$); C compared with D, E compared with F, C compared with E ($p < 0.001$); grouped GM compared with grouped JP, grouped HN compared with grouped KQ, grouped IO compared with grouped LR, grouped JP compared with grouped LR, grouped KQ compared with grouped LR ($p \leq 0.001$); G compared with J, H compared with K, I compared with L, J compared with L ($p \leq 0.015$); M compared with P, N compared with Q, P compared with R, Q compared with R ($p \leq 0.004$). All other combinations showed no significant differences.

Bivalve species	Algae species	Size class	N	Mean clearance rate L h^{-1}g $AFTM^{-1}$	Notations for significant differences		
Pacific oyster	*Isochrysis spp.*	1	14	0.31 ± 0.17	A	C	G
		2	17	0.41 ± 0.24			H
		3	15	0.23 ± 0.09			I
	Phaeodactylum tricornutum	1	18	1.94 ± 1.03		D	J
		2	9	1.59 ± 0.31			K
		3	15	1.16 ± 0.72			L
Blue mussel	*Isochrysis spp.*	1	13	0.79 ± 0.73	B	E	M
		2	16	0.71 ± 0.50			N
		3	11	0.67 ± 0.36			O
	Phaeodactylum tricornutum	1	12	2.45 ± 1.48		F	P
		2	8	2.13 ± 1.25			Q
		3	14	0.94 ± 0.91			R

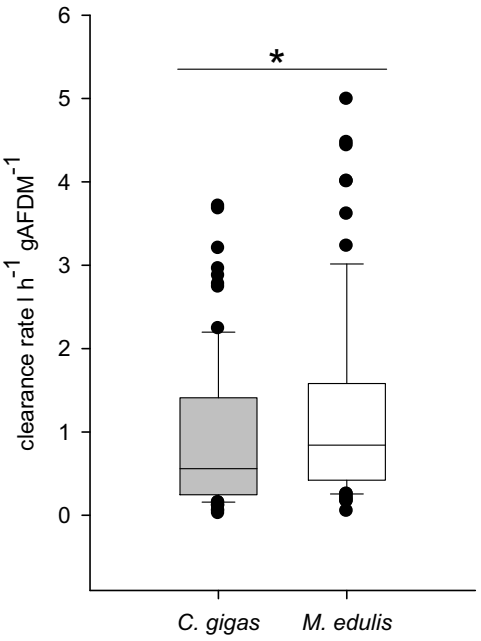

Figure 4: Clearance rate of Pacific oysters (N = 88) and Blue mussels (N = 74), independent of the algae species. Box plots show median (horizontal line within box), 25th and 75th percentiles (box) and 10th and 90th percentile (whiskers); circles indicate outliers. U-tests, * = $p < 0.05$.

When analysing the algae species separately, both bivalve species showed a similar filtration rate, which was significantly higher ($p < 0.001$) for *P. Tricornutum* than for *Isochrysis*. Yet for the smaller algal species *Isochrysis* Blue mussels showed a significantly ($p < 0.001$) higher filtration rate than Pacific oysters.

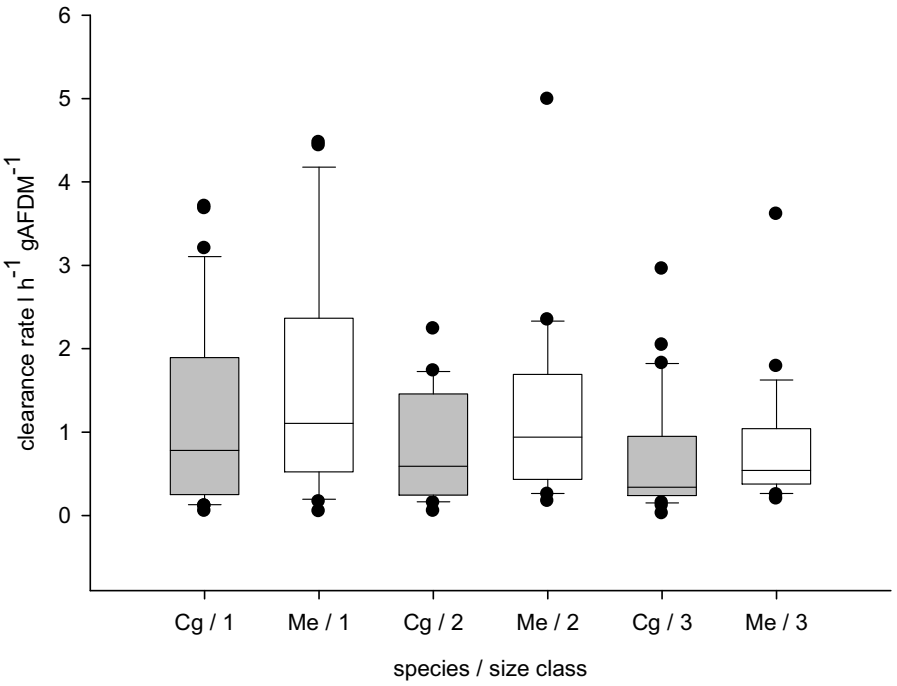

Figure 5: Box plots of clearance rates of Pacific oysters (Cg/1, N = 32; Cg/2, N = 26; Cg/3, N = 30) and Blue mussels (Me/1, N = 25; Me/2, N = 24; Me/3, N = 25). Numbers indicate size classes: Pacific oyster size class 1 = 48.3 mm ± 4.9, class 2 = 67.9 mm ± 4.7, class 3 = 112.5 ± 10.0 and Blue mussel size class 1 = 37.3 ± 2.5, class 2 = 47.6 ± 2.1, class 3 = 61.2 ± 3.5. Algae species were disregarded.

Discussion

Competition is an important mechanism, which influences the continued existence of species at a particular site if a resource is limited, particularly if a non-indigenous species is reproducing successfully in an area that is already occupied (Petren & Case 1996, Shea & Chesson 2002). As the non-indigenous Pacific oyster spreads out in the East Frisian Wadden Sea presently and occurs primarily on Blue mussel beds (Schmidt et al. 2008), competition for space and food between oyster and mussel was expected. Over the three study years, an increase in Pacific oyster densities was observed accompanied by a decrease of Blue mussel numbers. However, the decline of Blue mussels is generally observed in the East Frisian Wadden Sea and started before the expansion of the Pacific oyster (Herlyn & Millat 2004). On the investigated mussel bed oyster densities increased primarily in those areas where Blue mussels dominated before (Figure 2), which implies a potential competition for space between Blue mussel and Pacific oyster.

With the significant increase of the Pacific oyster and the significant decrease of the Blue mussel, our first hypothesis assumed that the oyster could potentially outcompete the mussel.

Figure 6: Blue mussels settling between Pacific oysters in a mixed oyster / mussel bed.

To the end of our investigation period, however, we found Blue mussels settling deep inside the structure of the oysters (Figure 6), which was also increasingly observed in 2006 (pers. com. Millat). Furthermore, Schmidt et al. (submitted) found that the population increase of the Pacific oyster had no negative influence on the Blue mussel population in the East Frisian Wadden Sea. The development of mixed oyster and mussel beds is reflected in the positive correlation of oyster and mussel densities in each year, with an increasing correlation coefficient from year to year. As well, additional investigations in 2005 strengthened the theory of a co-occurrence of oyster and mussel by a positive correlation of oyster and mussel abundances. Regarding the nascent mixed mussel / oyster beds, it seems that the oyster found a niche in the Wadden Sea ecosystem like other previously non-indigenous bivalves

(see also Reise et al. 2005) such as the clam *Mya arenaria* (Strasser 1999) and the razor clam *Ensis americanus* (Armonies and Reise 1999). However, there might still be an overlap between the ecological niches of Pacific oyster and Blue mussel with shared resources, such as food. The possibility of a formation of mixed mussel / oyster beds is shown by the occurrence of mixed mussel bed of *Perumytilus purpuratus* and *Semimytilus algosus* in Chile (Fernandez et al. 2000).

The spatial overlap and resulting proximity of oysters and mussels implies competition for the available food. The filtration experiments showed a slightly higher filtration rate for Blue mussels than for Pacific oysters (Figure 4), which was similar to the results of Deslous-Paoli et al. (1987), but contrary to the results of Troost et al. (2008). Factors that influence the filtration rate, such as water temperature (Jørgensen et al. 1990, Lee & Chin 1981, Walne 1972), food species that were used (Bayne et al. 1977, Newell et al. 1989), size and shape of the food (Lucas et al. 1987, Newell & Jordan 1983) and food concentration (Schulte 1975, Winter 1973) make comparisons with other studies difficult. Some studies found a similar filtration rate to ours (Lucas et al. 1987, Riisgård & Møhlenberg 1979, Ropert & Goulletquer 2000), but others found approximately 10 times higher filtration rates for both species (Kiorboe et al. 1981, Ropert & Goulletquer 2000, Walne 1972). The comparison of the filtration rate of oysters and mussels revealed an advantage in filtration efficiency for the mussel. This could explain why Blue mussels can cope with the vertical arrangement of oysters and mussels within the mussel bed and the resulting disadvantages, such as reduced food accessibility. Food depletion can occur above mussel beds in low current situations (Dame et al. 1984, Fréchette et al. 1989, Peterson & Black 1991). Oysters have their siphon higher in the water column and, thus, might have an advantage in reaching the food, however, the velocity of their inhalant feeding current is lower than in Blue mussels (Troost et al. 2004). Blue mussels living deeper inside the oyster bed can survive by their higher filtration rate. Thus, the lower filtration rate and the lower velocity of the inhalant feeding current of the oyster allow enough food to reach the Blue mussels.

Based on the findings presented here, the Blue mussel is not outcompeted by the Pacific oyster through competition for space, and both bivalves species can access enough food in the mixed mussel / oyster beds, which are likely to replace mussel beds in the tidal flats of the southern North Sea.

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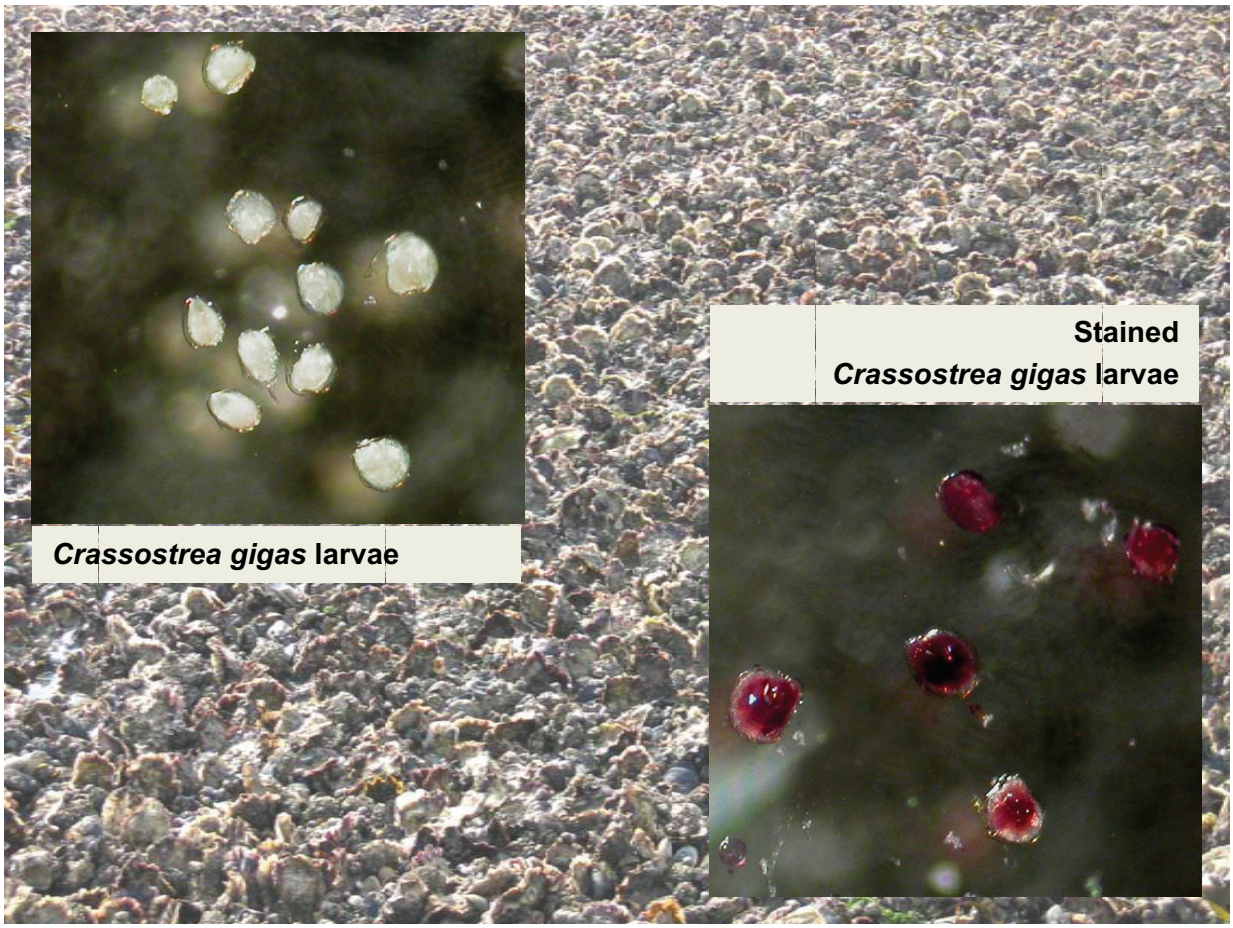
Chapter 4.1

Species identification of marine invertebrate early stages by whole-larvae in situ hybridisation of 18S ribosomal RNA



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Abstract

The ability to identify early life-history stages of organisms is essential for a better understanding of population dynamics and for attempts to inventory biodiversity. The morphological identification of larvae is time consuming and often not possible in those species with early lifehistory stages that are radically different from their adult counterparts. Molecular methods have been successful in identifying marine larvae; however, to date these methods have been destructive. We describe here an in situ hybridisation (ISH) technique that uses oligonucleotide probes specific for the 18S ribosomal RNA gene to identify marine larvae. Our technique leaves the larvae intact, thus allowing the description of larvae whose morphology was not previously known. Only 1 mismatch between the rRNA sequences of target and non-target species is sufficient to discriminate species, with nearly 100% efficiency. We developed a colourimetric assay that can be detected with a dissecting microscope, and is thus suitable for autofluorescent or large eggs and larvae that cannot be sorted under a microscope. Probe binding is revealed by an enzymatic reaction catalysed by either a horseradish peroxidase or an alkaline phosphatase. ISH was broadly applicable: it was effective in identifying eggs, larvae and adult tissues, soft-bodied larvae (polychaetes) and larvae with hard shells (bivalves), larvae belonging to different phyla and from different environments. Further advantages of this method are its relatively low cost, that only a minimal amount of equipment is needed, and that 100s of specimens can be processed quickly and simultaneously.

Keywords

Whole-larvae in situ hybridisation, Oligonucleotide probes, Species identification, Molecular ecology, Ribosomal RNA, Polychaetes, Bivalves

Introduction

Our understanding of the mechanisms governing population biology and dynamics, and our ability to predict changes in these populations and to manage marine invertebrate and fish species are complicated by their life cycles. Marine larvae are radically different from their adult counterparts, in morphology, habitat and mode of nutrition. During development, they undergo very rapid and extensive developmental changes. Moreover, larvae of a number of species are planktonic and have the potential to travel 100s to 1000s of kilometres transported by currents, to mix in the water masses with other species, before undergoing metamorphosis and beginning their adult life (Kinlan et al. 2005). Both adult benthic ecology and larval ecology need to be considered to develop a fuller understanding of the relative importance of larval and benthic dynamics to a species' spatial and temporal distributions, abundances and population structure (Eckman 1996). In many marine species, the first step towards larval ecology is merely to be able to identify early life stages in the environment.

Many marine invertebrate and fish larvae cannot be identified to the species level, either because closely related species are essentially identical morphologically, or because the larval forms of a species are unknown (Levin 1990). In species that have yet to be reared in the laboratory, which applies to the vast majority of all species, how does one match an unknown larva to a described species? In addition, there is an increasing realisation that the forms of many invertebrate larvae are very plastic and are determined by a number of environmental variables such as food (Sewell et al. 2004) and the physico-chemical properties of water (e.g. temperature in Shirley et al. 1987).

As an alternative to morphological methods, a number of biochemical and molecular methods have been developed (for review see Garland & Zimmer 2002). Immunological (Demers et al. 1993) and polymorphic allozyme electrophoresis techniques (Hu et al. 1992) have been used to discriminate larvae at the family or even the species level. However, environmental conditions, ontogenic changes in the larvae and sample preservation may alter protein concentration or conformation of the protein's epitope (Demers et al. 1993, Anderson et al. 1999). Larval proteins may also be highly conserved and may not differ sufficiently

among species to be used as species-specific markers. In addition, general antibody cross-reaction problems limit the resolution of immunological techniques (Garland & Zimmer 2002). Molecular methods using diagnostic DNA sequences isolated from adult specimens have been used successfully to identify larvae (Coffroth & Mulawka 1995, Morgan & Rogers 2001, Larsen et al. 2005), a number of which were based on ribosomal RNA sequences (Olson et al. 1991, Medeiros-Bergen et al. 1995, Comtet et al. 2000, Frischer 2000). Ribosomal RNAs (rRNA) are such excellent phylogenetic markers because they are extremely conserved in overall structure, allowing identification at higher taxonomic levels, and yet also highly variable in certain regions, allowing identification at the species level. To date, molecular identification methods have required DNA or RNA extraction and were therefore destructive.

Whole-cell in situ hybridisation methods using oligonucleotide probes targeting rRNA have successfully identified bacteria and archea (DeLong et al. 1989, Amann et al. 1990, Pernthaler et al. 2002), diatoms (Scholin et al. 1997), nanoflagellates (Lim et al. 1996), Microsporidia (Hester et al. 2000), ciliates (Petroni et al. 2002) and picophytoplankton (Simon et al. 2000). Despite the clear advantage of this technique for identifying larval stages, surprisingly, there has been only 1 attempt to extend it to marine larvae (Goffredi et al. 2006). These authors used fluorescent oligonucleotide probes to identify barnacle larvae by in situ hybridisation (FISH), but this method is limited by the strong autofluorescence of many marine eggs and larvae (Pradillon 2002), making it difficult to consider a general application of FISH for marine larvae.

We describe here a non-fluorescent method that uses horseradish peroxidase- or digoxygenin-labelled probes to which binding is revealed by a colour reaction. Coloured larvae can then easily be seen under a standard dissecting microscope while sorting plankton. This approach is similar to the one first described for bacterial cells (Amann et al. 1992). Here, we developed species-specific oligonucleotide probes targeting the 18S rRNA for invertebrate species in 2 different contexts where larval identification was needed for understanding dispersal processes. We targeted 4 polychaete species from a hydrothermal vent of the East Pacific Rise (*Alvinella pompejana*, *Alvinella caudata*, *Riftia pachyptila* and *Tevnia jerichonana*) and an introduced alien oyster species currently invading the southern North Sea

(*Crassostrea gigas*). In both cases, the aim was to identify a defined target species, among a mix of non-target species. By developing our method for use on such different larvae, i.e. polychaete larvae with a cuticle and bivalve larvae with a shell, we were able to show the broad applicability of our technique.

Materials and Methods

Sample collection

To design species-specific probes, sequences of all closely related species that occur in the same biogeographic range as the target species should be compared. At the time we designed probes for this study, only some of these sequences were available in GenBank. For this reason, in addition to specimens of the target species, closely related species were collected to obtain 18S rRNA gene sequences. Adult specimens of 9 polychaete species, including our 4 target species, were collected from hydrothermal vents between 9° N and 21° S on the East Pacific Rise (EPR) during cruises from 1994 to 2004 (Table 1). Upon reaching the surface after the submersible ascent, individuals were directly stored in liquid nitrogen or in 96% ethanol. *Crassostrea gigas* and 3 additional bivalve species were collected around the island of Sylt (Germany) in 2001 and stored in 70% ethanol (Table 1).

In situ hybridisation (ISH) assays were performed on larvae obtained from cultures, on eggs collected from adult specimens, or on adult tissues. *Crassostrea gigas* larvae were obtained from Guernsey Sea Farms. They were preserved in 70% ethanol in seawater and had been kept for up to 4 yr when used in ISH. *C. gigas*, *Ostrea edulis* and *Mytilus edulis* adult specimens were collected from an intertidal mussel bed south of the island of Juist (Germany). Tissues were preserved in 70% ethanol in seawater and kept up to 1 yr before ISH was performed. For deep-sea polychaete species, larvae were not available, so we used eggs collected from fresh specimens. They were preserved in several different ways: stored in 96% ethanol, fixed for 4 h to 2 d in 4% paraformaldehyde (PFA) in seawater and stored in phosphate-buffered saline (PBS: 145 mM NaCl, 1.4 mM NaH_2PO_4, 8 mM Na_2HPO_4, pH 7.5):ethanol (50:50), or fixed for 4 h to 2 d in 3% formalin in seawater and stored in 70% ethanol in seawater. Eggs had been preserved for up to 3 yr when used for ISH.

Table 1. Polychaete and mollusc specimens from which 18S rRNA sequences were obtained and used for probe design. EMBL: European Molecular Biology Laboratory; EPR: East Pacific Rise.

Species	No. of individuals	Tissue	Origin	EMBL accession number
Alvinella pompejana	2	Gills	9° N / EPR, 2000	AM159573
	1	Gills	17° S / EPR, 2004	
	1	Gills	21° S / EPR, 2004	
Alvinella caudata	1	Gills	EPR, 1994	AM159574
	1	Gills	17° S / EPR, 2004	
	1	Gills	21° S / EPR, 2004	
Paralvinella grasslei	2	Body wall	13°N / EPR, 2002	AM159575
Paralvinella pandorae	2	Entire body	17° S / EPR, 2004	AM159576
Hesiolyra bergi	1	Body wall	13°N / EPR, 2002	AM159577
Amphisamytha galapagensis	1	Entire body	14° S / EPR, 2004	AM159578
	1	Entire body	17° S / EPR, 2004	
Nereis sandersi	1	Body wall	21° S / EPR, 2004	AM159579
Riftia pachyptila	1	Eggs	17° S / EPR, 2004	AM159580
Tevnia jerichonana	1	Eggs	17° S / EPR, 2004	AM159581
Crassostrea gigas	1	Adductor muscle	Sylt Island	AM182263
Macoma balthica	1	Foot muscle	Sylt Island	AM182265
Ensis americanus	1	Foot muscle	Sylt Island	AM182264
Cerastoderma edule	1	Foot muscle	Sylt Island	AM182262

In order to assess the influence of the developmental stage on *in situ* hybridisation in polychaetes, we used embryos and larvae of a shallow-water species, *Platynereis dumerilii*, since vent larvae were not available. *P. dumerilii* larvae were obtained from laboratory cultures (Prof. A. Dorresteijn, Giessen University) at different developmental stages from 4 h old embryos to 6 d old juveniles. They were either stored in 96% ethanol or fixed for 4 h in 4% PFA in PBS and washed in ethanol:PBS (50:50). These larvae had been preserved for up to 1 yr when used for ISH.

18S rRNA sequences

DNA samples from polychaete and bivalve adult specimens were obtained using the method described by Zhou et al. (1996). Tissues were digested with Proteinase K, and DNA was recovered after a standard chloroform-isoamyl alcohol extraction procedure, precipitation in isopropanol, washing in ethanol, and resuspension in sterile-filtered water. As genetic

differentiation was shown using the COI mitochondrial gene in several vent polychaete species across their geographic distribution range (Hurtado et al. 2004), specimens originating from distant sites were selected, when available, to check for 18S rRNA intraspecific variability (Table 1). PCR amplification of the 18S rRNA genes was performed using 4 sets of primers (Table 2). A >1600 bp fragment of the 18S rRNA gene was amplified from DNA of *Alvinella pompejana*, *A. caudata*, *Paralvinella grasslei*, *P. pandorae*, *Amphisamytha galapagensis*, *Nereis sandersi*, *Riftia pachyptila* and *Tevnia jerichonana* using the primer combination 1f/2023r, from DNA of *Crassostrea gigas*, *Ensis americanus* and *Macoma balthica* using the primer combination Univ15f/Univ1765r, and from *Cerastoderma edule* using the universal primers developed by Sogin (1990). A ≈1400 bp fragment was amplified from DNA of *Hesiolyra bergi* by using the primer combination 1f/1486r. Each PCR contained 10 µl of 10× Eppendorf *Taq* buffer, 71 µl of H_2O, 25 µM of each dNTP, 150 mg BSA l^{-1}, 1 U of Eppendorf *Taq* polymerase, each primer at 0.5 µM, and 1 µl DNA template. PCR amplification was initiated by a 5 min denaturation step at 96°C, followed by 30 cycles of 94°C for 1 min, 51°C (for primer pairs 1f/2023r or 1f/1486r), 53°C (for primer pair Univ15f/Univ1765r), or 64°C (for universal primer pair from Sogin 1990) for 1 min and 72°C for 2 to 3 min; a final elongation step was performed at 72°C for 10 min. Amplified DNA was purified with a QIAquick PCR purification kit (Quiagen). Additional internal primers were designed for sequencing reactions (Table 2). Sequencing reactions were carried out on both strands, using the ABI BigDye prism dideoxy sequencing dye terminator kit and an ABI PRISM 3100 generic analyser (Applied Biosystems). Sequence data were edited with Sequencing Analysis software (Version 3.7, Applied Biosystems) and Sequencher 4.5 (Gene Codes Corporation). Sequences were submitted to GenBank, and accession numbers are given in Table 1.

Table 2. Primers used for the PCR amplification and sequencing reaction of the 18S rRNA genes.

Primer	Sequence 5'–3'	Treatment	Source
1f	CTG GTT GAT YCT GCC AGT	PCR amplification and sequencing	Winnepenninckx et al. (1995)
Univ 15f	CTG CCA GTA GTC ATA TGC	PCR amplification and sequencing	Frischer (2000)
Univ f	CAA CCT GGT TGA TCC TGC CAG T	PCR amplification and sequencing	Sogin (1990)
1486r	ACC AAC TAA GAA CGG CC	PCR amplification and sequencing	Present study
2023r	GGT TCA CCT ACG GAA ACC	PCR amplification and sequencing	Modified from Winnepenninckx et al. (1995)
Univ 1765r	ACC TTG TTA CGA CTT TTA	PCR amplification and sequencing	Frischer (2000)
Univ r	CTG ATC CTT CTG CAG GTT CAC CTA C	PCR amplification and sequencing	Sogin (1990)
429f	AGG GTT CGA YTC CGG AG	Sequencing (polychaetes)	Present study
915f	TTT GAA AAA ATT AGT GTG YTC	Sequencing (polychaetes)	Present study
1373f	TAA TTT GAC TCA ACA CGG G	Sequencing (polychaetes)	Present study
1854f	CAC ACC GCC CGT C	Sequencing (polychaetes)	Modified from Winnepenninckx et al. (1995)
505r	GTG GGT AAT TTG CGC G	Sequencing (polychaetes)	Present study
987r	RAR GTC CTI TTC YAT TAT TCC	Sequencing (polychaetes)	Present study
361f	ATC AGG GTT CGA TTC CGG	Sequencing (*Macoma balthica)*	Present study
570f	GCC AGC AGC CGC GGT	Sequencing (bivalves)	Frischer (2000)
919f	GAT TAA GAG AGA CTG CCG	Sequencing (*Crassostrea edule)*	Present study
1138f	GAA ACT TAA AGG AAT	Sequencing (bivalves)	Frischer (2000)
570r	ACC GCG GCT GCT GGC	Sequencing (bivalves)	Frischer (2000)
1138r	ATT CCT TTA AGT TTC	Sequencing (bivalves)	Frischer (2000)
1145f	AAT TGA CGG AAG GGC ACC	Sequencing (*Ensis americanus)*	Present study
1216r	ACC GGG TGA GGT TTC CCG	Sequencing (*M. balthica)*	Present study

Probe design

Probes were designed using the package software ARB (Ludwig et al. 2004). All 18S rRNA gene sequences of polychaetes and bivalves available in online databases at the time of the study, as well as the 13 sequences obtained in this study, were imported and aligned in

the ARB database. Alignments were manually corrected. For species for which we obtained 18S rRNA sequences from several individuals originating from distant populations, these were always 100% identical over the total length analysed. This indicates that our speciesspecific 18S rRNA probes could not have produced false negative identification caused by intra-specific variation.

Species-specific probes were designed using the PROBE-DESIGN function of the ARB software. They were named after the first letter of the genus and species name and the position targeted on the 18S rRNA gene. Probes were chosen to have at least 1 mismatch with any non-target species, and they were assigned so that the species used as a reference for the specificity test were available. For example, *Alvinella pompejana* probes were chosen so that the species presenting the most similar sequence at the target site was *A. caudata*. In some other regions, the 18S rRNA sequence was more similar to *Paralvinella grasslei* or *P. pandorae*, 2 other alvinellid species present at EPR vent sites. Since eggs of these 2 species were not available for the specificity test, probes in such regions were discarded. Probes were also designed to minimise selfcomplementarity and loop formation, which was checked using the OLIGO Primer Analysis software (Molecular Biology Insights). Potential complimentarity with non-target organisms whose sequences were not available at the time the probes were designed was checked again in June 2006 using the BLAST function of online databases against all published sequences.

Whole larvae in situ hybridisation

For ISH, probes were labelled with 2 different haptens: (1) horseradish peroxidase (HRP) (Biomers); and (2) digoxygenine (DIG) (Thermo Electron).

ISH was usually conducted in 1.5 ml tubes. However, with particularly fragile, rare, or tiny larvae, for which accidental pipetting while changing buffers had to be strictly avoided, all steps were conducted under a binocular dissecting microscope in 4-well Nunclon plates. Eggs, larvae, or tissues were first rehydrated in a graded series of ethanol in PBS. Then, different permeabilisation procedures were evaluated: 0.02, 0.05, 0.1, or 0.2 M HCl for 10 min at room temperature (RT); 0.1, 0.25, or 0.5% sodium dodecyl sulphate (SDS) for 15 min at

RT; 1, 10, or 100 µg Proteinase K ml^{-1} with Tween 0.1% for 1 to 30 min at 37°C or 10 min to 3 h at RT; 1 mg collagenase ml^{-1} for 10 min at 37°C with Tween 0.1%; 0.05, 0.1, 0.2, or 0.5% acetic acid for 15 min at RT with Tween 0.1% (detailed protocols are available upon request). After washing in PBS, hybridisation buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 8], 0.02% [w/v] SDS, 10% [w/v] dextran sulphate, 1% [w/v] blocking reagent [Boehringer], 0 to 60% [v/v] formamide [Fluka]) and 125 pg $µl^{-1}$ HRP-labelled probe or 250 pg $µl^{-1}$ DIG-labelled probe were pipetted onto the larvae. Overnight incubation (12 to 16 h) at 46°C was then carried out. Unspecific binding was removed by stringent washing in buffer with 14 to 900 mM NaCl, 20 mM Tris-HCl (pH 8), 5 mM EDTA (pH 8) and 0.01% (w/v) SDS, at 48°C. Stringency in the washing buffer was modulated through NaCl concentration, according to the formamide concentration in the hybridisation buffer (Pernthaler et al. 2001).

For hybridisation with HRP-labelled probes, a final wash in PBS was performed at RT. Probe binding was revealed by the addition of a solution of 1.25 mM TMB (3, 3, 5,5 tetramethylbenzidine; Research Diagnostics), which is a substrate oxidised by the HRP. In positive hybridisations a blue colour developed within 20 min at RT. Colour intensity was evaluated by eye. It varied from very light blue to very dark, and results were recorded according to an arbitrary scale of 8 levels (very light, light, medium-light, medium, mediumstrong, strong, dark and very dark).

For hybridisation with DIG-labelled probes, after washing the unbound probe, larvae were blocked in PBS-0.5% (w/v) blocking reagent for 30 min at RT, and then incubated with an anti-DIG–AP (alkaline phosphatase) antibody (1.5 U ml^{-1}, Fab fragment, Roche) in 100 mM Tris-HCl, 150 mM NaCl and 1% (w/v) blocking reagent overnight at 4°C. Unbound antibody was removed in a 30 min wash in PBS, and 2 × 5 min washes in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) at RT. The antibody was detected by incubation in NBT/BCIP staining solution (Roch) diluted 1:50 in 100 mM Tris-HCl and 100 mM NaCl. Red-purple colour developed after 10 to 180 min, and the colour reaction was stopped with TE-buffer (10 mM Tris-HCl, 1 mM EDTA). Colour intensity was evaluated by eye, and arbitrarily scaled from light red to dark purple.

Successive ISH was conducted with different HRP probes on eggs. After the first ISH, eggs were washed in PBS for 30 min and in a high-stringency (without NaCl) washing buffer for 1 h. TMB incubations were performed to check that no HRP probe remained, and eggs were washed again in PBS before incubation in hybridisation buffer with the new probe.

Images of ISH were recorded with a Nikon Coolpix 995 digital camera mounted on the binocular microscope, using the same exposure settings for each picture.

DNA extraction and PCR amplification from eggs and larvae

In order to assess the number of false positive and false negative results in ISH, DNA was extracted from a single egg or larva after ISH using the procedure described by Schizas et al. (1997), and the 18S rRNA gene was amplified and partially sequenced using the procedure described above. After ISH, eggs were washed in PBS before DNA extraction. This procedure was performed on 18 *Riftia pachyptila* eggs and on 6 *Platynereis dumerilii* larvae.

Results

Probe design

In Alvinellidae, the 18S rRNA sequences of the 2 *Alvinella* species examined in this study differed by at least 3% over 1792 bp, and by at least 4 and 5% over >1650 bp from all other alvinellid 18S rRNA sequences available (*Paralvinella grasslei*, *P. pandorae*, *P. palmiformis)*. In addition, *Alvinella* sequences have several large insertions in their 18S rRNA, unique to each species. These insertions provided highly specific sequences, with no or very low identity to sequences of non-target species, and were therefore chosen for the species-specific *Alvinella* probes. These probes differed by at least 3 base pairs from the target sequences in other alvinellid species (Table 3).

In Siboglinidae, despite the overall high identity (between 98 and 99%) between their 18S rRNA genes, we identified short sequence stretches that were sufficiently unique to *Riftia pachyptila* and *Tevnia jerichonana* to serve as target sites for speciesspecific probes. Both species are present at EPR vent sites, and their 18S rRNA genes are more similar to each other than to all other siboglinids, with 99% identity over 1783 bp. The chosen target

sequences had at least 1 unique base pair when compared with representatives of other siboglinid species (e.g. RP158; Table 3) and in most cases at least 2 base pair differences.

Table 3. Oligonucleotide probe sequences specific for 4 vent polychaete species (*Alvinella pompejana*, *A. cuadata*, *Riftia pachyptila*, *Tevnia jerichonana*) and for the oyster *Crassostrea gigas*.

Probe	Target organisms	Probe sequence 5'-3'	Tm (°C)	Target sequence 5'-3' in target species and closest non-target species		Reference
AP176	*Alvinella pompejana*	ACCAACGACAACTACCACG	58	CGUGGUAGUUGUCGUUGGU .U....CC.GCCTAC...G	(*A. pomejana*) (*A. caudata*)	Present study
AP1420	*Alvinella pompejana*	AGGACCACGGGCACACTG	60	CAGUGUGCCCGUGGUCCU U...U.......U.....	(*A. pomejana*) (*A. caudata*)	Present study
AC175	*Alvinella caudata*	AGTAGGCAGGACCAAGGC	58	GCCUUGGUCCUGCCUACU C..G.........GCGA.	(*A. caudata*) (A. pomejana)	Present study
AC1455	*Alvinella caudata*	GCCTGCCCTCCCACCTG	60	CAGGUGGGAGGGCAGGC GC...........C...	(*A. caudata*) (*A. pomejana*)	Present study
RP158	*Riftia pachyptila*	GCTCACGCGGTCGGAAC	58	GUUCCGACCGCGUGAGC ....C....	(*R. pachyptila*) (*T. jerichonana*)	Present study
RP1752	*Riftia pachyptila*	CGACCTCTAAGCCGTCAA	56	UUGACGGCUUAGAGGUCG .....C.U..........	(*R. pachyptila*) (*T. jerichonana*)	Present study
TJ202	*Tevnia jerichonana*	CGAACGACGCACCGATTG	58	CAAUCGGUGCGUCGUUCG ....C........C	(*T. jerichonana*) (*R. pachyptila*)	Present study
CG773	*Crassostrea gigas*	CATTGTACAGGCGAAGCG	56	CGCUUCGCCUGUACAAUG ..AA.......C.....	(*C. gigas*) (*Ostrea edulis*)	Present study
Cg1543	*Crassostrea gigas*	AGAATTACACACCCCAAT	50	AUUGGGGUGUGUAAUUCU .........C......A.	(*C. gigas*) (*O. edulis*)	Present study
CG1546	*Crassostrea gigas*	GGGAGAATTACACACCCC	56	GGGGUGUGUAAUUCUCCC ....C........A....	(*C. gigas*) (*O. edulis*)	Present study
EUK 516	Eukarya	ACCAGACTTGCCCTCC	52	GGAGGGCAAGUCUGGU		Amman et al, 1990
Non EUB338	Negative control	ACTCCTACGGGAGGCAGC	60			Wallner et al, 1993

For *Crassostrea gigas*, the most closely related co-occurring bivalve species in the North Sea are *Ostrea edulis* and *Mytilus edulis*. Their 18S rRNA sequences are, respectively, 97 and 92% identical to the *C. gigas* 18S rRNA over >1750 bp. *C. gigas*-specific probes were designed by targeting sequences exhibiting at least 2 base pair differences to *O. edulis* and *M. edulis* (Table 3).

Specificity tests

Designed probes were evaluated using eggs collected on adult specimens for polychaete vent species and using larvae obtained from culture and adult tissues for *Crassostrea gigas*.

Specificity was determined in a series of hybridisations with increasing formamide concentrations, causing an increase in stringency. These series were performed with HRP-labelled probes for each target species and with DIG-labelled probes for *C. gigas*. Specificity of any given probe did not differ between HRP- and DIG-labelled probes. Examples of such series are given in Fig. 1. With the HRP-labelled probe RP158, which is specific for *Riftia pachyptila*, we showed that even only 1 base pair difference is sufficient to discriminate the target species from other closely related species (Fig. 1a, see also Fig. 3c,d).

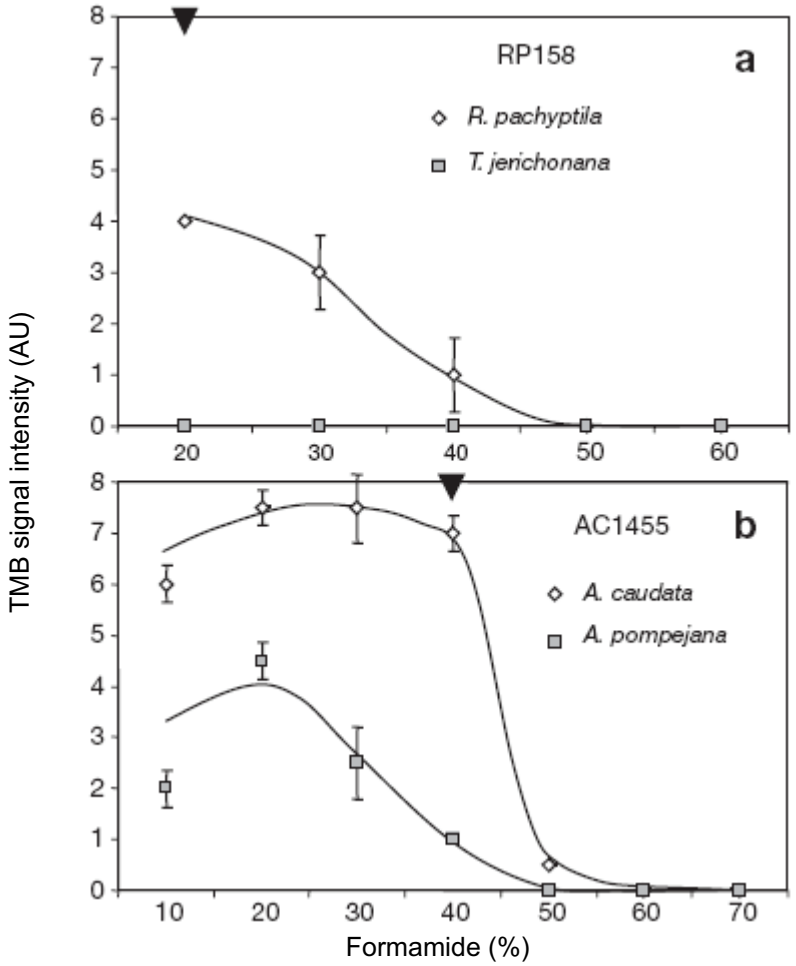

Figure 1: Comparison of the melting curves derived from the whole-egg hybridisation for the duplex between a specific horseradish peroxidase (HRP) probe and the 100% complementary target sequence and mismatched target sequence, with increasing formamide concentration as measured by the tetramethylbenzidine (TMB) signal intensity (AU: arbitrary units). (a) Duplex between RP158 and the *Riftia pachyptila* sequence (complementary) and the *Tevnia jerichonana* (1 mismatch). (b) Duplex between AC1455 and the *Alvinella caudata* sequence (complementary) and the *A. pompejana* sequence (3 mismatches). Optimised formamide concentrations are indicated by the black arrowheads. Error bars = ± SD

Stringent hybridisation conditions were evaluated for each designed probe. Each probe allowed the discrimination of the target species without producing false positives among the non-target organisms tested (Figs. 2 to 4, Table 4). For each assay, nearly 100% of the eggs of the target species were positively identified. The rare unstained individuals were damaged, and, in those cases, we could expect a loss of target ribosomes.

All probes did not give equally intense signals under stringent conditions (see Figs. 2 & 3). Probes AP1420, AC1455, RP1752, CG1543 and CG1546 showed stronger signals than probes AP176, AC175, RP158, TJ202 and CG773.

Table 4: ISH experiments demonstrating specificity of the probes, with formamide (FA) concentration in the hybridisation buffer required for specific ISH. For non-target species, the number of mismatches is indicated in parentheses.

Probe	Expected specificity	%FA	Signal demonstrated with:							
			Alvinella pompejana oocytes		*Alvinella caudata* oocytes		*Riftia pachyptila* oocytes		*Tevnia jerichonana* oocytes	
AP176	*A. pompejana*	20	+		-	(10)	-	(no match)		
AP1420	*A. pompejana*	30	++		-	(3)	-	(no match)		
AC175	*A. caudata*	20	-	(6)	+		-	(no match)		
AC1455	*A. caudata*	40	-	(3)	++		-	(no match)		
RP158	*R. pachyptila*	20	-	(8)			+		-	(1)
RP1752	*R. pachyptila*	40	-	(7)			++		-	(2)
TJ202	*T. jerichonana*	20	-	(11)			-	(2)	+	
			Crassostrea gigas larvae & tissue		*Ostrea edulis* tissue		*Mytilus edulis* tissue			
CG773	*C. gigas*	10	+		-	(3)	-	(4)		
CG1543	*C. gigas*	10	++		-	(2)	-	(2)		
CG1546	*C. gigas*	10	++		-	(2)	-	(3)		

Effect of fixation

We tested whether the use of different fixation methods would influence the ISH reaction using eggs of the vent polychaetes. Ethanol-fixed eggs always showed a strong hybridisation signal, as well as eggs fixed with formalin or paraformaldehyde for a few hours. However, paraformaldehyde fixation times of >24 h resulted in low or undetectable hybridisation signals.

Increasing permeabilisation time, or the use of more concentrated permeabilisation solutions did not improve ISH in specimens fixed in paraformaldehyde for >24 h.

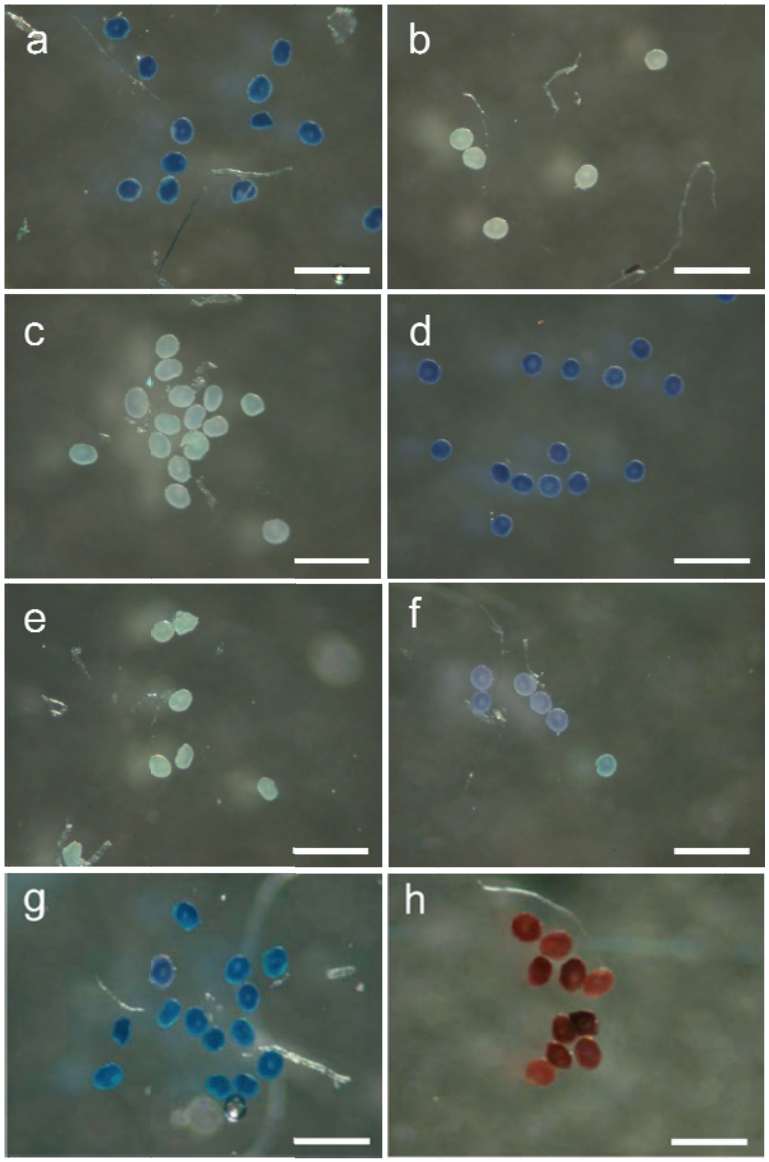

Figure 2: *Alvinella pompejana* and *A. caudata*. Evaluation of specific probes. Blue colour indicates hybridisation with HRP probe. Reactions were performed under stringent conditions, as defined in Table 4, with *A. caudata* (a,c,e,g,h) and *A. pompejana* (b,d,f) eggs using specific probes: AC1455 (a,b), AP1420 (c,d) and AP176 (e,f); AC1455 after a first in situ hybridisation (ISH) with AP1420 (g). Panel (h) shows hybridisation with the general eukaryote probe EUK516 labelled with digoxygenin as indicated by the red-purple colour. Scale bars = 500 µm

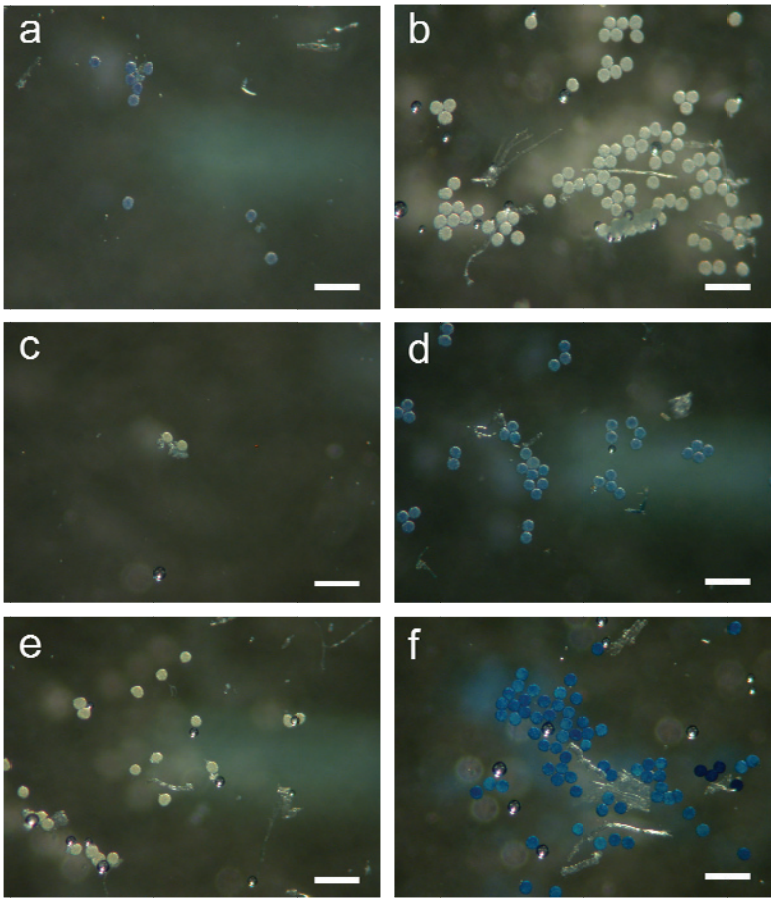

Figure 3: *Riftia pachyptila* and *Tevnia jerichonana*. Evaluation of specific probes. Blue colour indicates hybridisation with HRP probes. Hybridisation reactions were performed under stringent conditions, as defined in Table 4, with *T. jerichonana* (a,c,e) and *R. pachyptila* (b,d,f) eggs using specific probes TJ202 (a,b), RP158 (c,d) and RP1752 (e,f). Scale bars = 500 µm

Effect of permeabilisation

Permeabilisation procedures were adapted to the type of structure surrounding the larvae. *Crassostrea gigas* larvae are protected by their calcite shell. Efficient permeabilisation was achieved by using HCl at relatively high concentrations (0.1 M) (Fig. 4d). In polychaetes, the cuticle develops in early larval stages (Hausen 2005). In oocytes of vent species and in early embryos of *Platynereis dumerilii* (4 h embryos) no permeabilisation was required (Fig. 5a), although a short incubation in 0.02 M HCl resulted in a more homogeneous colouration. For larvae older than 1 d (trochophore stage), HCl treatment was not efficient (Fig. 5b). Increasing HCl concentration or incubation times resulted in a heavy loss of morphology.

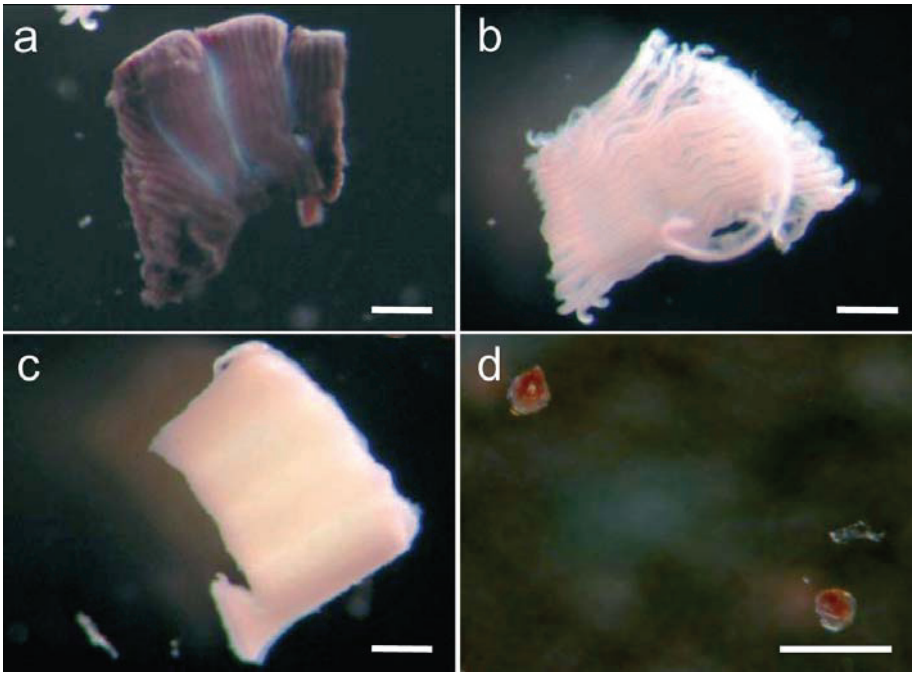

Figure 4: *Crassostrea gigas*. Evaluation of specific probe CG1546 using DIG probes. The deep-red staining indicates hybridisation with the probe: (a) *Crassostrea gigas* gill tissue; (b) *Mytilus edulis* gill tissue; (c) *Ostrea edulis* gill tissue; and (d) *C. gigas* larvae. Scale bars = 500 µm

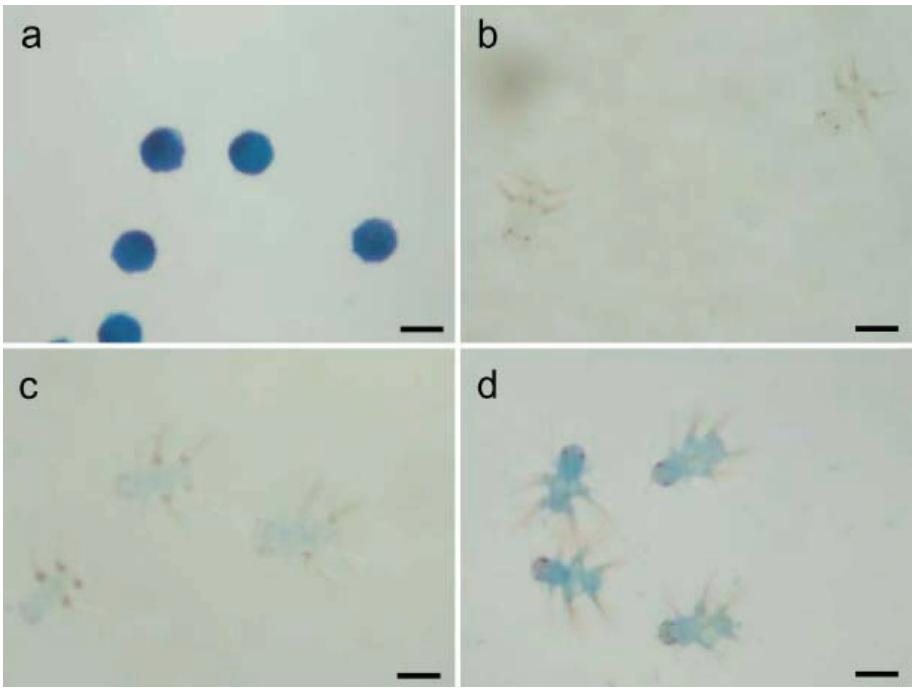

Figure 5: *Platynereis dumerilii*. *In situ* hybridisations at different developmental stages: (a) HCl (0.02 M)-treated 4 h old embryos, EUK516; (b) HCl (0.02 M)-treated 6 d old larvae, EUK516; (c) Proteinase K (10 µg ml^{-1}, 20 min, with Tween)-treated 3 d old larvae, EUK516; and (d) SDS (0.5%)-treated 6 d old larvae, EUK516. Scale bars = 150 µm

Proteinase K is commonly used in embryo and larva permeabilisation protocols. We used it in concentrations varying from 1 to 100 µg ml^{-1}, with incubation times varying between 5 min and 3 h. Signal intensity obtained with this permeabilisation treatment was always weak (Fig. 5c), and the highest Proteinase K concentrations resulted in a complete loss of signal and ultimately a loss of morphology. Permeabilisation with collagenase, acetic acid, or a combination of both did not increase the signal intensity (data not shown). Finally, best results for polychaete larvae were achieved using 0.5% SDS (Fig. 5d). Optimal permeabilisation and hybridisation procedures are summarized in Table 5.

Table 5. Summary of steps for ISH with marine eggs and larvae with HRP- or DIG-labelled oligonucleotide probes. All steps are conducted at room temperature except when specific temperature is mentioned (see 'Materials and methods' for details). ON: over night

Stage	Eggs and early embryos	Soft-bodied polychaete larvae	Hard-shell bivalve larvae
Permeabilisation	• Rehydrate in a graded series of ethanol in PBS		
	• Incubate 10 min in 0.02 M HCl (facultative) • Wash in PBS (facultative)	• Incubate 20 min in 0.5% SDS • Wash in PBS	• Incubate 10 min in 0.1 M HCl • Wash in MilliQ water • (1) Wash in 70% ethanol and let dry at RT, or (2) wash in PBS
Hybridisation	• Add 30 µl (1.5 ml tube procedure) or 300 µl (plate procedure) hybridisation buffer containing the probe at 125–250 pg µl–1 • Incubate ON (12–16 h) at 46°C • Wash 3 × 40 min in washing buffer at 48°C Wash in PBS 10 min		
Antibody reaction (only for DIG-labelled probes)	• Incubate in 0.5% blocking reagent in PBS 30 min • Incubate with antibody solution ON at 4°C • Wash in PBS 30 min Wash in TBS 2 × 5 min		
Probe binding visualisation	<u>HRP-labelled probes:</u> • Incubate in TMB staining solution maximum 45 min • Observation <u>DIG-labelled probes:</u> • Incubate in NBT/BCIP staining solution maximum 3 h • Stop colour reaction with TE buffer Observation		
Post-hybridisation (tested only for HRP-labelled probes)	• Wash in PBS 30 min • Wash in high stringency washing buffer and proceed with new ISH		• Not tested

Post-ISH analysis

Environmental samples usually include a mix of larvae from different species, and we therefore examined if it is possible to identify >1 species by hybridizing eggs or larvae several times successively with different species-specific HRP-labelled probes. Using eggs of vent species and *Platynereis dumerilii* larvae, we found that early larval stages can go through ISH procedures at least 2 times successively without loss of morphology. Between hybridisations, eggs or larvae have to be washed with a high-stringency washing buffer (without NaCl) in order to remove the attached probe from the first ISH. Probe signal intensity did not vary significantly whether a species-specific probe was applied at the first or at the second hybridisation (Fig. 3g).

In some cases it may be desirable to analyse larval genes using PCR, for example to validate probe identification of larvae, or to examine genes besides 18S rRNA for additional phylogenetic information. We extracted DNA from single *Riftia pachyptila* eggs and single *Platynereis dumerilii* larvae after they had been hybridised. The 18S rRNA gene could be amplified by PCR in 16 (89%) of the *R. pachyptila* eggs and all *P. dumerilii* larvae. Sequencing of the first 700 base pairs confirmed that there were no differences in the 18S rRNA sequences of specimens examined with and without ISH treatment. This method thus allows further examination of ISH-treated specimens using PCR=based methods.

HRP or DIG probes?

In order to evaluate the ISH procedure on natural plankton samples that may include considerable amounts of sand, algae and other debris, *Crassostrea gigas* larvae were mixed with plankton samples collected around the island of Juist. Unspecific blue background labelling of debris was observed, sometimes making it difficult to pick out larvae in the sample. We therefore developed an alternative protocol using a DIG probe combined with an AP-labelled anti-DIG antibody instead of the HRP probe. Since the kinetics of the reaction catalysed by AP are much slower than those catalysed by HRP, background labelling did not develop, or only after several hours. This time lapse is then sufficient to sort the larvae. DIG-labelled probes were also used successfully with polychaete eggs (example in Fig. 2h).

Discussion

All molecular methods developed so far for species identification in larval stages have been destructive, preventing further analysis of the larvae, which would be valuable for those that have not yet been described (Garland & Zimmer 2002), such as hydrothermal vent larvae. The whole-larvae colourimetric ISH method presented here allows the identification of larvae to the species level, without damaging morphology (however, ultrastructural details of the larval shell in bivalves that are examined by scanning electron microscopy and used for species identification might be lost after HCl treatments). For each species, we were able to develop probes that bound specifically to their target with nearly 100% efficiency, and without producing false positives with closely related species, even when target and nontarget sequences differed by only 1 mismatch. By making slight changes in the permeabilisation steps, we showed that the ISH method is effective with eggs, as well as with larvae and with adult tissues. The colourbased assay produced a bright blue or red signal, according to the labelling system used. Although not tested here, the simultaneous use of 2 probes labelled with each of the 2 haptens would allow 2-colour ISH assays in which 2 species could be simultaneously identified. Compared to fluorescent methods, such colour methods are better suited to be used with a standard dissecting microscope, where the bright signal produced by the probe hybridisation can easily be distinguished, and large amounts of plankton can be efficiently sorted.

ISH identification assays must meet the challenge of designing probes to discriminate among sequence differences at the species level, while retaining insensitivity to polymorphism within the target species. The 18S rRNA gene evolves slowly and has been used to resolve deep branching orders among different orders and families of organisms including invertebrates (Winnepenninckx et al. 1995, Bleidorn et al. 2003). It usually does not vary at the species level, and in some cases does not differ between closely related species. Here, even in families where 18S rRNA sequences are highly similar such as the siboglinid tubeworms, we showed that it is still possible to design species-specific probes based on single mismatch discrimination between target and non-target species (Fig. 1a). Since the 18S

rRNA gene has both regions that are highly conserved and highly variable, probes can be targeted to signature sites characteristic for species, genera, families, or orders (Amann et al. 1990). Within mixed environmental samples where one has no precise idea of the potential species present in the sample, nested approaches can be carried out by successively applying probes specific to the lower and to the higher taxonomic level. In addition, the conserved nature of the 18S rRNA gene at the species level makes it suitable for identifying individuals over a broad geographical range. Another advantage of the 18S rRNA gene is the fairly large database of sequences available, allowing the design of probes for a wide range of species and comparison with a maximum of non-target sequences.

In groups where the 18S rRNA gene evolves so slowly that not even 1 base pair difference can be used to discriminate the target species, other ribosomal genes could be used. The 28S rRNA gene, which is longer than the 18S rRNA gene, may potentially provide a higher number of probe binding sites (Peplies et al. 2004). The mitochondrial 16S rRNA gene could also be used, since mitochondrial genes are known to evolve more rapidly than nuclear ones. Finally, genes such as the mitochondrial cytochrome *c* oxidase subunit I (COI) have been proposed as good candidates for species identification, because this gene has a high inter-specific variability together with low intra-specific variability (Hebert et al. 2003). However, when using non-rRNA sequences to design probes for ISH methods, further methodological developments are required, since mRNA is much less abundant and stable than rRNA.

The design of a good probe also depends on its binding efficiency, which is influenced by its target site in the rRNA gene. It was previously shown that the 16S rRNA of Bacteria and Archaea, and the 18S rRNA of Eukarya (*Saccharomyces cerevisiae*) are not equally accessible to probe binding (Behrens et al. 2003). Certain domains, such as the sequence stretch at Positions 585 to 656 (*Escherichia coli* numbering), are consistently inaccessible to probe binding in prokaryotic 16S rRNA and in eukaryotic 18S rRNA. Similarly, the probe CG773 targeting the corresponding area in *Crassostrea gigas* 18S rRNA gave a weak signal, adding evidence that this region of the 18S rRNA gene should be avoided when designing new probes. Our ISH experiments also showed that all probes targeting the 5'-end of the 18S

rRNA gene (AP176, AC 175, RP158, TJ202) gave relatively low signals in the target species. Behrens at al. (2003) predicted a rather weak accessibility in the corresponding region in *S. cerevisiae*. On the other hand, we also found that the probes targeting the 3'-end of the gene (AP1420, AC1455, RP1752, CG1543, CG1546) gave a rather strong signal. In this case, our pattern does not completely fit data from Behrens et al. (2003), since AP1420 and AC1455 target areas with rather low predicted accessibility; whereas RP1752, CG1543 and CG1546 target areas with medium to high predicted accessibility. However, data from Behrens et al. (2003) also showed that even a slight shift along the rRNA sequence can produce a very strong increase in the probe signal.

ISH assay efficiency and sensitivity also strongly depend on the preservation and permeabilisation treatments. Preservation with cross-linking fixatives such as formalin or PFA should never exceed a few hours, because they tend to reduce considerably the probe penetration to the target molecules. A negative effect of formalin fixative has also been reported for ISH on diatoms (Miller & Scholin 2000).

Successful ISH depends strongly on the initial permeabilisation steps, in particular when HRP-labelled probes are used. Depending on the type of structure surrounding the larvae, permeabilisation has to be adapted. In eggs and very early embryos, cell membrane and fertilisation envelope might be relatively easy to permeabilise, whereas in older stages, which have developed cuticles or shells, much stronger permeabilisation procedures might be required.

The optimal permeabilisation depends on the species and also on the life stage of the larvae. Treating a mixed sample of larvae from the environment with a single permeabilisation method might leave some larvae impermeable and result in false negative results. With strong permeabilisation, softer larvae might lose their integrity and target rRNA, again producing false negatives. Prior to the use of an ISH assay, minimum sorting based on general morphology is helpful but does not require specific taxonomic expertise. Once this initial step is performed, larvae can be rapidly processed using ISH, and identified. We showed that ISH assays do not prevent the subsequent use of other methods. If necessary, post-hybridisation checks for false

positives or negatives might be performed using methods based on DNA extraction and amplification.

Despite the relatively elevated cost of HRP probes compared to mono-labelled fluorescent probes, the total cost of 1 hybridisation assay was 0.94 Euros when performed in plates, and 0.12 Euros when performed in 1.5 ml tubes. DIG-labelled probes are cheaper than HRP probes, but higher concentrations are required for a sensitive result, and subsequent antibody detection increases the total cost of the assay. The cost of a DIG assay performed in plates is 2.1 Euros, and 0.23 Euros when performed in 1.5 ml tubes. Overall, considering that a large number of individuals (several 10s or even 100s in plate assay) can be processed in 1 single assay, ISH methods can be performed with minimum expense. Besides, very little equipment is required: only a standard dissecting microscope and a hybridisation oven are necessary to perform the assay. This method is thus well suited to be used on board during survey field trips.

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Chapter 4.2

Pacific oyster identification by whole-larvae *in situ* hybridisation – improved method of Pradillon et al. (2007) – for the direct use in a plankton sample

Spawning of a group of Pacific oysters in Pendrell Sound (Quayle 1969).

Abstract

Studying larvae ecology requires processing large amounts of meroplankton samples, good expertise of the morphology of the investigated species group, and the required time to do so. The method described here allows processing a large amount of samples in a short time and without morphological identification skills of the target species group. This makes it easier to investigate species with a large distribution range (e.g. dispersal of a cosmopolite), or to identify invasive species, which occur only sporadically in the habitat at the start of an invasion.

Keywords:

Larvae identification, *in situ* hybridisation, plankton, bivalves, *Pacific oyster*

Introduction

Invasions of alien species occur more and more frequently and gain increased influence in marine environments (Carlton 1989, Carlton & Geller 1993, Occhipinti-Ambrogi & Savini 2003, Ruiz et al. 1997). Identification of non-indigenous species during transport and further dispersal in a recipient region could be important for prevention and management of invasions as well as the understanding of invasion mechanisms, like the invasion of the Pacific oyster (*Crassostrea gigas*) into the Wadden Sea, Germany. Species identification to species level is likewise essential for many other issues of ecology. Especially the identification of benthic organisms during their larval stages is often difficult (Levin 1990). The dispersal of sessile benthic organisms mostly takes place during their mobile larval stage. In case of bivalves, it is very difficult to identify their larvae with morphological characteristics. An alternative way to identify species offers a number of biochemical and molecular methods (for review see Garland & Zimmer 2002). However, few of these methods meet the requirements of larval ecology studies, e.g. during dispersal, such as an bio-invasion, larvae are very scattered and patchily distributed, which requires a very extensive sampling and the processing of such large samples limits many investigations (Garland & Zimmer 2002). Garland & Zimmer (2002) described the requirements of a molecular marker technique to process samples in larval ecology studies: The technique should (1) involve no direct sorting of organisms from a sample (instead, the probe would be applied to a multi-species assemblage in a small dish); (2) be effective for intact, whole organisms (so that specimens can be saved for other analyses); (3) result in a sufficiently detectable surface expression on the organism for detection via image analysis techniques (for automated counting and sizing); (4) be relatively inexpensive to develop (to generate probes for a large number of species); (5) be relatively inexpensive to produce once developed; (6) produce accurate and repeatable results.

To understand the bio-invasion of Pacific oysters into the southern North Sea, the issue of how dispersal of the oyster within the Wadden Sea takes place is an important point. The Pacific oyster was introduced into the Oosterschelde estuaries (The Netherlands) for aquaculture purpose in 1964 (Drinkwaard 1999). In 1983, the Pacific oyster reached the Wadden Sea in the southern North Sea at the island Texel (Bruins 1983), from where it

spread throughout the southern Wadden Sea towards the Elbe-estuary (Dankers et al. 2004, Reise 1998, Schmidt et al. 2008, Wehrmann et al. 2000). The first Pacific oysters in the Lower-Saxony part of the German Wadden Sea were recorded in 1998 at the island Baltrum (Wehrmann et al. 2000). The dispersal of oysters into and within the Wadden Sea occurred through pelagic larvae drifting with water currents (Schmidt et al. 2008, Wehrmann et al. 2000). As the distance of larval dispersal can vary, larvae supply can also differ with potential consequences for communities, which can either facilitate or constrain bio-invasions (see Scheltema 1986, Todd 1998).

For a better knowledge about the dispersal pathways of Pacific oyster larvae, a method was developed for an easy identification of the larvae (Chapter 4.1). The method enables qualitative and quantitative investigations on larvae and other small species which are difficult to classify, while further morphological identification remains possible, as the specimens are left intact. The method is based on a whole-larvae *in situ* hybridisation of the 18S ribosomal RNA with a following detection of the larvae through a colour reaction (colourimetric *in situ* hybridisation, CISH), which enables the identification by using a binocular microscope. The aim was to identify the larvae directly in a plankton sample without prior separation of all bivalve larvae. Using the original method of Pradillon et al. (2007, Chapter 4.1) an unspecific colour reaction appeared in the plankton sample (see Figure 1), which complicated the quick identification of larvae without time-consuming prior separation. The aim of this investigation was to avoid the unspecific colour reaction.

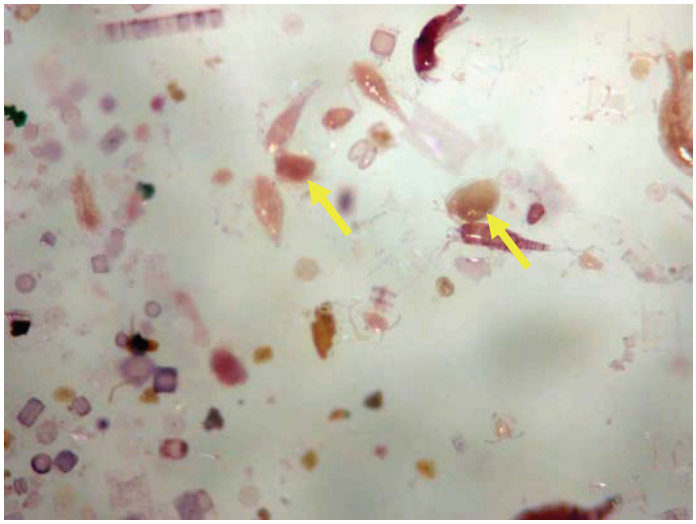

Figure 1: Pacific oyster larvae stained with whole-larvae colourimetric ISH method in a plankton sample. Other species were stained by unspecific colour reaction. Yellow arrows indicate the oyster larvae.

Methods

The plankton sample, including extra added Pacific oyster larvae (to ensure presence of oyster larvae in the sample) stored in 70 % ethanol (non-denatured) with seawater, were transferred into reaction tubes (for the development of the method 1.5 ml tubes were used) in which all following steps were done. For the permeabilisation of the calcite shells of the Pacific oyster larvae, HCL with a final concentration of 0.01 M up to 0.1 M (depending on the shell of the larvae, for *C. gigas* 0.1 M was used) was added and incubated at room temperature (RT) for 5 to 10 min (for *C. gigas* incubation was 10 min). To stop the process 1 ml of 70 % ethanol (non-denatured) with H_2O dd was added, centrifuged 10 to 15 sec and removed as far as possible. Before starting the probe hybridisation, the ethanol had to be evaporated (a climatic chamber at 37 °C can be used). For probe hybridisation 40 µl of hybridisation buffer with probe (formamide 0 % up to 70 %, NaCl 900 mM, Tris-HCl 20 mM, Blocking reagent 1 X (Roche, Penzberg, Germany), SDS 0.02 %, probe dilution from 1/200 to 1/400 end concentration (Stock concentration was 50 ng/µl)) was added (for *C. gigas* larvae 10 % formamide and 1/200 probe dilution was used), incubation was at 46 °C with shaking for 4 h to overnight (for *C. gigas* incubation overnight). After hybridisation the sample was washed in 1 ml washing buffer matching the previously used formamide concentration (for 0 % formamide NaCl 900 mM for 70 % formamide NaCl 7 mM, Tris-HCl 20 mM, EDTA 5 mM, SDS 0.01 %) at RT for 1 min., centrifuged for 10 to 15 sec. and the supernatant of the washing buffer was discarded, followed by a second washing step at 48 °C for 2 to 4 hours. After washing the samples were pre-incubated, before the incubation with Anti-DIG, at RT for 2 h in 1 ml PBS 1 X (10 X PBS: NaCl 1,37 M, KCl 0,027 M, Na_2HPO_4-$7H_2O$ 0,043 M, KH_2PO_4 0,014 M, pH 7,3) with 1 X Blocking reagent, after the incubation centrifuged for 10 to 15 sec. and the supernatant of the PBS / Blocking reagent buffer was discarded. Before the preparation of the Anti-DIG buffer, the Anti-DIG had to be pre-absorbed. For the pre-absorbtion a non-used aliquot (200 µl) of the plankton sample was used. The aliquot was washed in 1 ml PBS 1 X at RT for 1 min., centrifuged for 10 to 15 sec. and the supernatant of the washing buffer was discarded, followed by a second wash with 1 ml H_2O milliQ at RT for

1 min., centrifuged for 10 to 15 sec. and the supernatant H_2O was discarded. For the pre-absorption 1 ml H_2O milliQ with anti-DIG (working concentration of 1:100 to 1:500) was added and incubated for 1 h at RT, centrifuged for 10 to 15 sec. The H_2O supernatant was used for the preparation of the Anti-DIG buffer. For incubation with Anti-DIG 100 µl Anti-DIG buffer was added (Tris-HCl 100mM, NaCl 150 mM, Blocking reagent 1 X) with a Anti-DIG working concentration of 1:100 to 1:500 (concentration is 1500 to 7500 mU/ml of Anti-Digoxigenin-AP, Fab fragment) the incubation was at RT for 2 h to 4 h or overnight at 4 °C. After incubation with Anti-DIG the sample was washed in 1 ml PBS 1 X with 0.5 X Blocking reagent for 1 h at RT, centrifuged for 10 to 15 sec. and the supernatant of the PBS / Blocking reagent was discarded. After the washing with PBS two washing steps with TBS (Tris-HCl 0.05 M, NaCl 0.15 M, pH 7.5) followed for 5 min. at RT. For the colour reaction the larvae were transferred on a glass slide to let the TBS evaporate. For the colour reaction 20 to 25 µl NBT / BCIP staining solution was added (10 ml staining solution contains 200 µl of NBT / BCIP Stock Solution (Roch, Penzberg, Germany) and Tris-HCl 0.1 M, NaCl 0.1 M). After an incubation of 10 to 180 min. a blue to purple colour will be developed. The Colour reaction can be stopped with TE-buffer (Tris-HCl 10 mM and EDTA 1 mM).

Results and Discussion

For species identification, which is essential in many issues of ecology (Levin 1990), a molecular method was developed (Chapter 4.1). With this method the dispersal pathways of Pacific oyster larvae should be investigated, but the method yet fulfilled not all necessary requirements as described by Garland & Zimmer (2002). The improved method presented here (chapter 4.2) includes all necessary requirements and from Garland & Zimmer (2002) described properties. The greatest innovations are the ability to identify the target species directly in a plankton sample and the preservation of the morphology, as all previous methods (see Garland & Zimmer 2002) were destructive. The difficulty with the whole-larvae colourimetric ISH method was the use of the method directly in a plankton sample, which at first resulted in an unspecific colour reaction. First trails with horseradish peroxidase (HRP) - labelled oligonucleotide probes for the identification of oyster larvae in plankton samples

showed no unspecific colour reaction on other species, but lead to a staining of soil particles (results not shown), which is not acceptable especially if this method should be used for an automated detection. HRP-labelled oligonucleotide probes can be used in plankton samples as they do not contain such an amount of soil particles as plankton samples from the Wadden Sea, particularly because it is the cheaper method (see chapter 4.1). With the use of digoxygenine (DIG) - labelled oligonucleotide probes no stained soil particles appeared anymore, but a prolonged colour reaction lead to non-specific staining. This problem also occurred in a method described by Le Goff-Vitry et al. (2007). The problem with the DIG anti-DIG system, which leads to the unspecific staining, was a non specific docking to wrong epitopes by the anti-Dig, and was resolved with the improved method, a pre-absorption of the anti-Dig (this chapter). With the improved method no unspecific colour reaction appeared and the Pacific oyster larvae could be detected easily in the plankton sample (Figure 2), thereby the best results were attained when using the probe CG1546 (Table 1).

Figure 2: Pacific oyster larvae stained with the improved whole-larvae colourimetric ISH method in a plankton sample. Yellow arrows indicate the oyster larvae, other coloured particle are e.g. lacquer splinter or the more brownish are soil particles.

Table 1: Oligonucleotide probe sequences for the oyster Crassostrea gigas (see chapter 4.1)

Probe	Target organism	Probe sequence 5'–3'	Temp (°C)	Target sequence 5'–3' in target species
CG1546	*Crassostrea gigas*	GGGAGAATTACACACCCC	56	GGGGUGUGUAAUUCUCCC

Using the method attention should be drawn on the permeabilisation step as mentioned in chapter 4.1, because a too intensive treatment with HCl leads to a destruction of the specimens. The problem here is that most bivalve larvae specimens have closed shells after fixation and the HCl concentration for permeabilisation could only be adjusted for a restricted size class of larvae. The HCl concentration can be reduced with application of a muscle relaxing substance as it is described in Le Goff-Vitry et al. (2007), where a solution of $MgCl_2$ was used before fixing the larvae to allow the adductor muscles to relax. This leaves the shell open for an easier permeabilisation and subsequent hybridisation procedure. Regarding the list of required attributes for a molecular method (see above Garland & Zimmer 2002), the described whole-larvae colourimetric *in situ* hybridisation method is a tool which enables investigations of a multitude of studies of marine invertebrate and fish larvae.

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General Discussion

Bio-invasions become an increasing problem in marine systems and can have, in case of a successful invasion, lasting impact on the recipient ecosystems as well as on the economics of the region. Therefore, it is important to know how marine bio-invasions occur. Many studies in context of marine bio-invasions deal with the issue of the vector, the current state of distribution in the recipient region or the impact on the ecosystem. However, only few studies deal with spreads by natural means of a non-indigenous species, after arriving in a region where it can prosper. One example are the investigations on the invasive northern Pacific seastar *Asterias amurensis* (see Nimpis 2002a), e.g. Ross et al. (2004) conducted a field experiment to investigate the combined effects on the community by two introduced marine predators, the northern Pacific seastar and the European green crab *Carcinus maenas*, because the spatial overlap was imminent in Tasmania.

In this case study the initial natural spread and the potential impact of the non-indigenous Pacific oyster *Crassostrea gigas* in the East Frisian Wadden Sea (Germany) was investigated (see Figure 1). For this purpose the population dynamics of the Pacific oyster in the entire East Frisian Wadden Sea were investigated over a three year period, the potential impact on the native Blue mussel was analysed and a genetic method for species identification was developed that allows continuing investigations on the larval dispersal of the Pacific oyster.

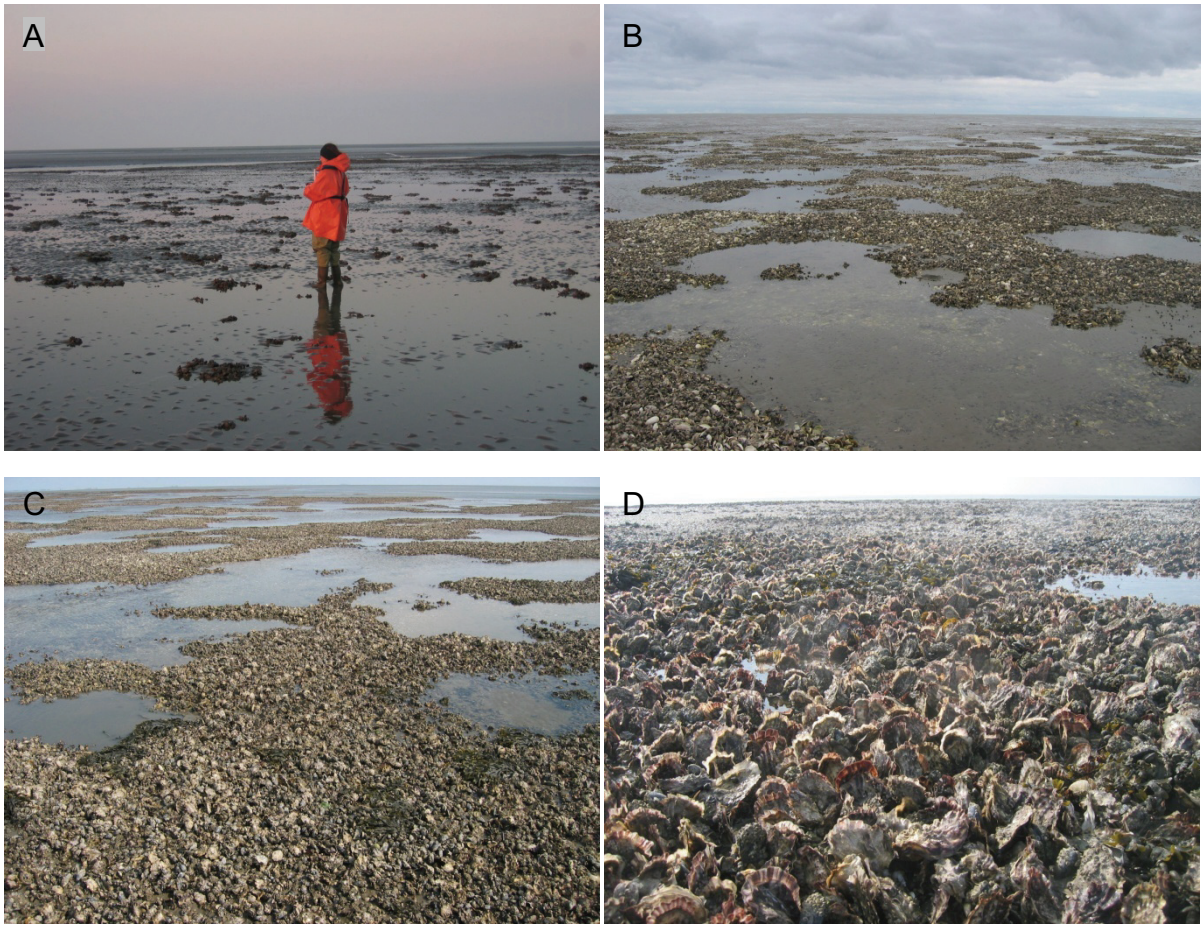

Figure 1: Different types of Pacific oyster beds in the East Frisian Wadden Sea. A: scattered occurrence can be found on the edge of a bed; B and C: different shapes and densities of most occurring beds; D: densely populated oyster bed.

Population dynamics of the Pacific oyster in the East Frisian Wadden Sea

Prerequisite characteristics which the oyster should have for the development of populations in new regions

The Pacific oyster can cope with a wide variety of environmental conditions which facilitates the world wide spread of this species (see Nimpis 2002b). The invasion success of the oyster is probably due to: the tolerance for different temperatures and salinities (Neudecker 1985, Reise 1998), being a hard substrate inhabitant (Arakawa 1990, Quayle 1969) but able to settle on shells in softer substrates (Dankers et al. 2004, Diederich et al. 2005), and an r-selected life history (Neudecker 1985, Quayle 1988). With this attributes, the

Pacific oyster has the characteristics of a successful invader, following criteria given by Ricciardi & Rasmussen (1998, Table1).

Table 1: Criteria for a successful invader and corresponding attributes of the Pacific oyster

Criteria for an successful invader	**Attributes of the Pacific oyster**	**Reference**
Abundant and widely distributed in original range	The oyster occurs natural from north Japan to Korea	Nimpis 2002b
Wide environmental tolerance	Wide tolerance for different temperatures and salinities	See Nimpis 2002b
High genetic variability	Several strains exist from the Pacific oyster	Quayle 1969
Short generation time	Appearance of a new cohort every year in the Wadden Sea during the study period	This study
Rapid growth	Growth in the Wadden Sea is approximately 40 $\pm$ 15 mm^{-y}	This study
Early sexual maturity	Sexual maturity is reached after one year	See Nehring 2006
High reproductive capacity	Females produce 20-100 million eggs	See Nehring 2006
Broad diet (opportunistic feeding)	The oyster feeds on phytoplankton and protists	Dupuy et al. 1999, Raillard & Menesguen 1994
Gregariousness	The oyster can build up dense populations after a short time	This study
Possessing natural mechanisms of rapid dispersal	With the planktonic larval stage the oyster has the ability for a rapid dispersal	This study
Commensal with human activity (e.g., ship ballast-water transport)	Many introductions worldwide for aquaculture purpose	Chew 1990

Considering the characteristics of the Pacific oyster and the attributes for invasive aquatic species, this may explain the successful spread of the Pacific oyster worldwide as well its invasion into the southern North Sea (this study, Reise 1998, Dankers et al. 2004, Diederich et al. 2005).

Population development and increase of the Pacific oyster in the Wadden Sea

Once a species has invaded a new region, the increase in abundances may lead to the development of a stable population following some typical phases (Essink & Dekker 2002, Reise et al. 2006). The first phase is the settlement phase with the species arriving in the recipient region (Ribera & Boudouresque 1995). In this phase the non-indigenous species must reach a minimum number of individuals which enable the establishment of a reproducing population (Ashton & Mitchell 1989, Macarthur & Wilson 1967). Often there is a time lag between arrival and population increase, this can be rather variable between areas as well as between species (Crooks & Soulé 1999), which was shown by the history of the Pacific oyster invasion in different regions of the world (see Chapter 1). For example, in Chile it took 5 years before the first wild oysters were found after introduction (Orensanz et al. 2002), and in South Africa even 51 years (Robinson et al. 2005). During this time lag and with a smooth transition to the establishment phase a steady increase may lead to a stable equilibrium density (Essink & Dekker 2002). For the Pacific oyster invasion in the Wadden Sea an establishment phase of 4 to 6 year is assumed (see Chaper 1). This time variation can be related to differences in population growth between the different areas in the Wadden Sea (Chapter 1), whereas other conditions such as temperature are nearly the same in the entire Wadden Sea (Chapter 2). Thus, some oyster populations might reach a stable equilibrium density and some not. After the population is established, the population growth of many invasive species changes into a subsequent exponential growth (Williamson 1996). Furthermore, oyster populations with a high population increase can work as a seedbed which ensures offspring for new recruitment events. So far, the presumed population development for the Pacific oyster in the East Friesian Wadden Sea follows the typical phases of an invasion (see Figure 2). The investigations of this study were conducted during the early expansion phase of the oyster in the East Frisian Wadden Sea (Figure 2).

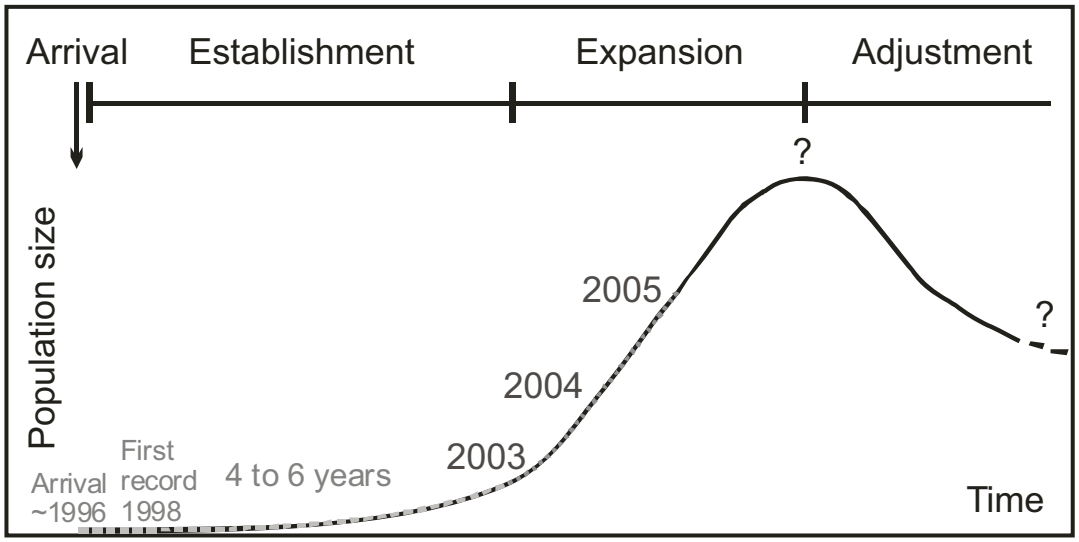

Figure 2: Schematic model for the invasion of the Pacific oyster into the Wadden Sea of Lower Saxony (Germany), showing presumed population development and the chronological integration of our study (Figure changed after (Reise et al. 2006))

The expansion phase can end in a very high and steady population density (Essink & Dekker 2002), or the population can decline to a lower level (Parker et al. 1999). On this level or close to the maximum level the population can attain a stable population size (Ribera & Boudouresque 1995). Calculations of the population growth revealed a population growth for the Pacific oyster (Chapter 1) similar to those of native bivalve populations in the East Friesian Wadden Sea. This is indicative for a population size evolving similar to one of a native bivalve population. Considering that the Pacific oyster increase started from a small propagule stock, however, a similar population growth as a native population might lead to a high population size and hence, the oyster could become the most abundant epibenthic bivalve species in the Wadden Sea. That the Pacific oyster is able to reach very high abundances shows the oyster invasion in France (Christian & Morgane 2007).

Larvae dispersal

Progress has been made in the understanding of larval developmental patterns and the complexities of larval habitat selection and metamorphic behaviour (Young 1990). Larval supply, development and spread are important for the development and preservation of bivalve and many other marine and terrestrial populations. In addition, species-specific behaviour of planktonic larvae can explain patchiness in adult distributions (Grosberg 1982, Hannan 1981). In case of the invasion biology there is an interest in the knowledge of how an invasive species spreads in a new region to develop a prediction model to assess the influence of a newly detected invasive species in the recipient region. First of all, such a model is helpful for control and management of bio-invasions and particularly to prevent them before an invasive species can harm the recipient region (see Bax et al. 2001, Thresher & Kuris 2004). As a basis for such a model a combined data set of population dynamics data and data of the larvae supply and spread are important. The investigation of population dynamics of the Pacific oyster in this study revealed details about the population spread in the beginning of the invasion, which was previously unknown for benthic invasive species, and helped to gain knowledge about how larvae disperse in the Wadden Sea (Chapter 1, and see below). For further investigations on larval dispersal (Chapter 4) a genetic tool was provided by this study to identify invertebrate and fish larvae in plankton samples.

The larvae supply, development and spread of bivalve larvae, as well as post-settlement processes until successful recruitment (see Ólafsson et al. 1994) underlie several processes and influences of biotic and abiotic nature. Thereby, larval supply may have been the limiting factor for oyster population growth (Cardoso et al. 2007), whereas larvae transport and behaviour might regulate the spread of the population. A summary of processes and factors during pre-settlement, settlement and post-settlement that are likely to determine benthic community structure is listed by Todd (1998). If conditions allow the production of offspring, pre-settlement processes are important for the larval supply and spread (Table 2).

Table 2: Processes and factors (see Todd 1998) influencing Pacific oyster larvae supply and spread in the Wadden Sea.

Pre-settlement processes influencing larvae supply and spread	**Occurring in the Wadden Sea and important for the Pacific oyster**
Predation on larvae (in the water column)	In the Wadden Sea through planktotrophic fish and jellyfish
Oceanographic influences (e.g. directional currents, upwelling, Ekman transport)	There is no direct influence in the North Sea, because the North Sea is an epeiric sea
Local hydrographic effects (e.g. tidal current variation, residual drift, hyposaline runoff)	Tidal currents have an influence on the Wadden Sea and a residual current exist from west to east along the southern North Sea coast (see Chapter 1 and Wehrmann et al. 2000)
Larval 'quality' (are all larvae equal?)	Larval quality for larvae in the North Sea is not investigated yet, but a different is to expect between the larvae origin from the two introduction sites in the Oosterschelde and at the island Sylt whereas oyster spat came from different countries (see Nehring 2006, Wolff & Reise 2002)
Larval behaviour (e.g. vertical migration, responses to salinity)	Oyster larvae have the ability for a directed swimming (see Troost et al. 2008b), and larvae can crawl around and release again from the substrate to find the best site for settlement (see Arakawa 1990).
Substratum electivity (making the 'correct' choice)	The oyster favours shells of their own species for settlement (see Chapter 2 and Arakawa 1990)
Benthic predation (e.g. inhibitory effects of adult suspension feeders)	Filter feeder can feed on oyster larvae (see Troost et al. 2008a)

An estimation of larvae supply and spread could give the number of larvae in the water column and their dispersal in the area. Pacific oyster dispersal occurs during the planktonic larval stage, when larvae are transported passively by water currents. The spread of invasive bivalves through currents is known from other bio-invasions, like the spread of the invasive mussel *Mytilus galloprovincialis* in South Africa (Mcquaid & Phillips 2000). The main theory about the spread of the Pacific oyster into the East Frisian Wadden Sea describes also the dispersal by water currents, i.e. a residual current induced by wind and tidal currents (Wehrmann et al. 2000). The spread described by Wehrmann et al. (2000) is a spread over a long distance but theoretically possible (see Armonies 2001). Such a long distance spread occurs only under special weather conditions at the southern North Sea coast and without these conditions only a local spread is possible. Are larvae transported mostly within the tidal flat area or also offshore in front of the island? Samples were taken along the cost before

(offshore) and behind the back barrier islands each year during the spawning season. On every sampling site for quantitative analysis 100 litres were filtered through a 55 µm plankton net and stored in 70 % ethanol (for genetic analysis) with seawater. These samples still have to be analysed with the genetic species identification method developed in this study (Chapter 4). If the larvae do not disperse over a long distance, how far would they move away from a mussel bed? First insights into larval retention in the vicinity of oyster reefs come from the population dynamics data. From the second year onwards, the increase of Pacific oyster abundances was higher on some mussel beds than on other, adjacent mussel beds, which could be due to a local recruitment event i.e. larvae did not move away from the origin mussel bed (see discussion in chapter 1). To investigate this, meroplankton samples should be taken around a single mussel bed over the spawning season.

However, to analyse the abundance and dispersal of larvae, it is important to identify the larvae to species level, which is often very difficult for meroplanktonic larvae. This explains why larval ecology remains poorly documented and prevents the full understanding of dispersal and colonisation processes. Especially for bivalve larvae the species identification is difficult due to the morphological similarity of early larval stages. Molecular genetics offers a solution for easy species identification and in chapter 4 a method is described which enables the identification of Pacific oyster larvae directly in a plankton sample. The speciality of this method is the preservation of morphology, which allows counting of the species in the sample to determine the abundance and it enables a control of the identification by the morphological character of the species. Furthermore, the DNA will be not destroyed so additional genetic analyses can be conducted for e.g. further population genetic studies.

Further spread in the North Sea region – what makes the Pacific oyster a successful invader?

The worldwide spread of the Pacific oyster (see Ruesink et al. 2005) shows the success of the oyster as a ‘global player’, being distributed by aquaculture and moreover by natural means with subsequent establishment of wild populations (e.g. Andrews 1980, Chew 1990, Korringa 1976, Quayle 1988). The potential of the Pacific oyster to invade a new region is furthermore reflected in the spread into more northern regions, to Denmark, Norway and

Sweden (Chew 1990, Nehring 2006, Reise et al. 2005), although it was thought that the Pacific oyster would not be able to reproduce in the North Sea because of its natural adaptation to relatively warm waters (Drinkwaard 1999). Yet, the development of the Pacific oyster population in the North Sea (Dankers et al. 2004, Diederich et al. 2005, Drinkwaard 1999, Reise 1998 and this study see chapter 1) shows how the decision to permit the establishment of an aquaculture with a non-indigenous species lead to a lasting impact on the environment. Aquaculture can contribute to worldwide bio-invasion on a comparable scale to ballast water (Minchin and Gollasch 2002). Further dispersal and unintentional distribution will spread the oyster in the North Sea. For example, Dutch mussel farmers already found Pacific oysters among Blue mussel seed transported from the German to the Dutch Wadden Sea (Wolff and Reise 2002).

What makes the Pacific oyster so successful? One reason is the wide environmental tolerance, which might be linked to the broad genetic variability of the different strains of the Pacific oyster (see also Nehring 2006). These different strains have their origin in several areas with different abiotic environmental conditions (e.g. water temperatures) within the native distribution range. The genetic and / or physiological variation of the different strains can contribute to the successful adaptation of the Pacific oyster to the environmental conditions in the southern North Sea (see Hamdoun et al. 2003, Lee 2002, Pörtner and Knust 2007). Probably there was one strain imported to the Oosterschelde, providing the propagule population which invaded the Wadden Sea. The faster oyster population growth and spread between Lower Saxony compared to the northern Wadden Sea could indicate genetic and / or physiologic adaptation. Spread happens only by release of a new generation, and thus, only those specimens are involved in the spread which can survive in the environment which are genetically and / or physiologically adapted to it. Oyster farms like the one at Sylt are importing seed oysters from different sources (see also Table 2), resulting in a mix of several populations from different environments. Thus, the genetic pool of the northern Wadden Sea population near Sylt could be different to that of the East Frisian Wadden Sea population. This could possibly explain the differences in population dynamics of the North Frisian (Diederich et al. 2005) and East Frisian (Chapter 1) region (see also Lapègue et al. 2006). The genetic and

physiological aspects need further studies as well as the identification of the origin, the strain, of the 'wild' Pacific oyster populations in the North Sea.

Influence of the invasive Pacific oyster on the Wadden Sea ecosystem

Influence on the native Blue mussel

The bio-invasion of the Pacific oyster in the Wadden Sea could have a possible direct impact on the native Blue mussel, because the initial invasion of the oyster took place on Blue mussel beds (this study, Wehrmann et al. 2000), which are providing the main hard substrate in the Wadden Sea, yet other hard substrates are artificial. Therefore, the potential use of the same ecologic niche by the Pacific oyster and the Blue mussel might lead to competition for the same resources. To find evidence for a direct competition between oyster and mussel, the population dynamics, spatial distribution on a mussel bed and filtration rate of both species were compared (see chapter 2 and 3). The results show that mixed beds of oysters and mussels are the likely future scenario. A cross-section of a mixed oyster mussel bed (based on own observations) is shown in Figure 3.

Figure 3: Co-occurrence of Pacific oysters and Blue mussels on a mussel bed, sketch of a cross-section.

During the beginning phase (first year of this study) of the invasion the Pacific oyster seemed to displace the Blue mussel. Approximately three years after the significant increase in the oyster population (chapter 1), the Blue mussel settled more and more within the three dimensional structure developed by the oysters (chapter 3 and Millat, pers. com.). Diederich (2005b) discussed also the formation of mixed beds with a variation in abundances of the mussel depending on changing summer and winter temperatures. The temperature during this study (chapter 2) facilitated the population growth of the oyster (see also Diederich et al. 2005), while high summer and mild winters inhibited mussel recruitment (Beukema et al. 2001, Diederich 2005a, Strasser et al. 2001). There was no evidence for negative effects from the Pacific oyster population on the population of the Blue mussel (Chapter 2). More likely, the Blue mussel profits from the oyster due to the protection against predators in the three dimensional structure of an oyster reef. The low recruitment success of the mussel occurred after mild winters due to increased predation on mussel larvae (Strasser & Guenther 2001).

Yet another possibility for a spatial pattern is the development of a vertical zonation on the tidal flats where the oyster settles with a higher abundance in the lower intertidal and the mussel with a higher abundance in higher situated areas of the intertidal but with a smooth transition between oyster and mussel dominated beds. This hypothesis is based on own observations in the field and was similarly discussed by Diederich (2005). The development of vertical zonations as consequence of an exclusion is known from rocky shores. For example, the mussel *Mytilus edulis* settled on higher shore as a result of competition with *Mytilus galloprovencialis* at the coast of Washington (Suchanek 1981). Subtidal occurrence of the Pacific oyster in the Wadden Sea has not yet been documented, apart from single specimens found during dredging near Sylt (Diederich, pers. comment). No oysters were found when dredging in the west part of the study area (close to the island Juist) (own investigations).

Beside the spatial competition, competition for food is also conceivable between the Pacific oyster and the Blue mussel (see chapter 3). However, the results of the investigation on the filtration rate of both species revealed an advantage in filtration efficiency for the Blue mussel. Considering that mussels live deeper inside structure of a mixed oyster / mussel bed (Figure 3) and given the filtration rates of both species (Chapter 3), both species seem to have

the same possibility to reach available food, supporting a future scenario of mixed oyster / mussel beds.

The spatial proximity of oyster and mussel increases the possibility that free swimming larvae become ingested by adult filter feeders. Experiments with the Pacific oyster and the Blue mussel imply that larvae of both species become ingested by their adults, whereas oyster larvae become less filtrated than mussel larvae which is due to a higher swimming speed of the oyster larvae (Troost et al. 2008a, Troost et al. 2008b) Further it was also shown that there are differences in the filtration rate between oyster and mussel larvae (Troost et al. 2008a). Hence, it is not clarified if and how this affects the population dynamics of both species.

Further influence on the recipient Wadden Sea ecosystem

With their formation of reef structures in the soft sediment environment of the Wadden Sea the Pacific oyster provides a substrate and habitat which can offer other species shelter and an area for settlement (see Chapter 3). Hence, the oyster functions as ecosystem engineer in the Wadden Sea. Species that previously had only a slight opportunity to settle in the Wadden Sea, such as hard substrate inhabitants, now have the possibility to colonise the reefs. Furthermore, the reef structure of the Pacific oyster provides a better shelter (more free space) for many species that live in the Wadden Sea, e.g. for the shore crab *Carcinus maenas,* which can hide from predators (see Figure 4). One example of an invasive species which provides shelter for other species and, hence, had an impact on the recipient community is the introduction of the reef-building polychaete *Ficopomatus enigmaticus* into the Mar Chiquita coastal lagoon of Argentina (Schwindt et al. 2001). An indication for the habitat value of Pacific oyster reefs in the Wadden Sea was given by Görlitz (2005), who found higher abundances of mobile epibenthic species on oyster reefs compared to Blue mussel beds. These are only some of the conceivable influences on the Wadden Sea ecosystem which could arise through the invasion by the Pacific oyster. Crooks (2002) described that invaders which directly modify ecosystems exert the largest impacts with cascading effects for resident biota and facilitating further invasions. In the Wadden Sea, the hard substrate provided by the Pacific oyster directly modifies the ecosystem (see above).

Furthermore, the hard substrate provided by the oyster also facilitates the further dispersal of conspecifics (see chapter 2), an example is the dispersal on the edge of a mussel bed (see Figure 1A), where oyster clusters can be found (see Figure 2 of the Introduction). One example of an invasive species that was normally a hard substrate inhabitant but invaded soft sediments is the expansion of dreissenid mussels across sedimentary environments in western Lake Erie, North America (Berkman et al. 2000).

Figure 4: Shore crab hiding in the three-dimensional structure of the Pacific oyster.

The potential influence of increasing densities of Pacific oysters on the associated community of Blue mussel beds is not clear yet, nor whether there would be a change in the community associated with mixed mussel / oyster beds (Broekhoeven 2005, Görlitz 2005, Kochmann 2007). In the study of Görlitz (2005) the species richness did not differ significantly between Blue mussel beds and Pacific oyster beds. However, the species occurred with different abundances (Görlitz 2005), which led to the assumption that the dominance structure

of the associated community will change from the former Blue mussel towards the Pacific oyster beds (see also Smaal et al. 2005). This could result in a change of the functional relationships in the ecosystem. For example, the cryptic occurrence of mussels within oyster reefs could lead to a loss of mussels, as well as of the associated fauna of mussels beds, as a food source for birds (see below).

Another fact which could lead to a change in the ecosystem is the high abundance (Chapter 1) and biomass production (Chapter 2) of the Pacific oyster. On the one hand, the high density with the three dimensional structure favours the protection of prey species (see above) and may lead to a loss of the mussel bed as food source for various predators such as fish or birds foraging on mussel beds. Ricciardi et al. (1998) specified that in some cases high densities can become a pest to other species in the invaded community, e.g. the zebra mussel *Dreissena polymorpha*. On the other hand, the biomass of the oyster could provide food for other species, e.g. birds or crabs or, after the oyster died, for small organisms or microorganisms. First investigation with benthic predators (shore crab *Carcinus maenas* and starfish *Asterias rubens*) show that they prefer the Blue mussel as food, but they are also able to prey on the Pacific oyster (Diederich 2005b). This indicates that the Blue mussel may become of less relevance as a prey item to starfish who can switch to oyster as a new food source. The use of mussel beds as feeding site is also known for birds which feed on Blue mussels or the associated fauna of mussel beds (e.g. Nehls et al. 1997). First investigations (see Scheiffarth et al. 2007) suggest that for birds feeding on the associated fauna (e.g. Curlews *Numenius arquata,* which feed on shore crabs) no difference in habitat quality is to be expected. For birds feeding on mussels, the conversion of mussel beds to oyster beds is probably negative, but reactions are species specific. However, contrary to mussels, even small oysters cannot be detached from the substrate to be used as food by swallowing or breaking the shell. The highest influence of the oyster could occur on the mussel feeding Common Eiders (*Somateria mollissima),* which seem to be not capable to feed on oysters. However, other birds may adapt to the new food, as it was observed in oystercatchers (*Haematopus ostralegus)* feeding on oysters (Esser pers. com.), and is also indicated by studies elsewhere in the world (e.g. Butler & Kirbyson 1979, Tomkins 1947,

Tuckwell & Nol 1997). In general, feeding densities of birds on oyster beds are at present lower than feeding densities on mussel beds (Scheiffarth et al. 2007).

Conclusion

This case study during a beginning bio-invasion shows that the Pacific oyster has spread into the entire East Frisian Wadden Sea and reached a viable population size. The population growth of the oyster over the three study years was similar to a native bivalve population and increased from year to year, which demonstrated that the oyster was in the exponential growth phase of an invasive population. During the beginning invasion the distribution of oysters in the East Frisian Wadden Sea developed patchy, instead of an abundance gradient declining from the source population as found in the first study year, which might be due to larval dispersal and larval supply. To investigate this, a molecular method was developed to identify oyster larvae in meroplankton samples. Considering the population growth, the Pacific oyster could become the predominant epibenthic bivalve in the Wadden Sea. Investigations of abiotic conditions, such as the temperature, which are important for the growth of the oyster and therefore crucial for the spread, shows that the Wadden Sea is a suitable habitat for the invasion of the Pacific oyster and enables the oyster to establish self sustaining populations. As the Pacific oyster is a hard substrate inhabitant, Blue mussel beds provide the prevalent substrate for settlement in the soft sediment environment of the Wadden Sea during the initial phase of the invasion. During the further spread the oyster can provide their own settlement substrate and therefore have the ability to enlarge their population. The spread of the Pacific oyster on mussel beds may lead to competition between the oyster and the mussel. But the Pacific oyster did not outcompete the Blue mussel and had no negative effect on the population growth of the mussel. An influence in the Wadden Sea ecosystem generally is to be expected, which is indicated by the high biomass production and the habitat structure provided by the oyster.

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