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The ecological function of intertidal shellfish reefs for benthic algae

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DOI:
[10.33612/diss.182497420](https://doi.org/10.33612/diss.182497420)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2021

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
Andriana, R. (2021). *The ecological function of intertidal shellfish reefs for benthic algae*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen. <https://doi.org/10.33612/diss.182497420>

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**The Ecological Function
of
Intertidal Shellfish Reefs
for
Benthic Algae**

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The research presented in this thesis was carried out at the Benthic Ecology group at the Groningen Institute for Evolutionary Life Sciences of the University of Groningen.

This research was funded by the Indonesia Endowment Fund for Education (LPDP). Financial support for the printing of this thesis was received from the University Library and the Graduate School of Science and Engineering of the University of Groningen.

This thesis should be cited as : Andriana, R. (2021). The ecological function of intertidal shellfish reefs for benthic algae. PhD Thesis, University of Groningen, Groningen, The Netherlands.

Cover and layout Rosyta Andriana

Printed by RVV/Grafisch | www.rvvgrafisch.nl

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The Ecological Function of Intertidal Shellfish Reefs for Benthic Algae

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on
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*Untuk keluargaku terkasih –
jauh di mata, dekat di hati –
Berkah dalem.*

Bila orang hendak sungguh-sungguh memajukan peradaban, maka kecerdasan pikiran dan pertumbuhan budi harus sama-sama dimajukan

If people really want to advance civilization, then intelligence and growth of mind must be promoted together.

-Sulastin Sutrisno, Surat-surat Kartini: Renungan Tentang dan Untuk Bangsa-

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TELUK LANGSA 501-Class Tank Landing Ship, Indonesian Navy
 Marine Biology Field trip to Pulau Pari, Thousands Island, Jakarta –
 Faculty of Fisheries and Marine Science, Bogor Agricultural University
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Preface

Rosyta Andriana

My interest in marine ecology was started at my bachelor's, at the Faculty of Fisheries and Marine Science Technology, Bogor Agricultural University (IPB), West Java.

Here, I learned a lot of new and interesting things about freshwater and marine ecosystems; from a small lake – mangroves – to a complete marine tropical ecosystem.

A marine biology field trip facilitated by Indonesian's Navy, having the honor to travel on one of their Class tank Landing Ship, KRI Teluk Langsa 501, has given me a special and unforgettable experience as a young ecologist and has made me fall in love (deeper) to Marine Life.

Further, I am convinced to choose Marine Ecology as my field interest: during fieldwork, I learned that we are not only learning and discovering the interaction of organisms and their habitat, but also the interaction and cooperation among us, the researchers; which literally, the whole process forms a network of interaction.

Being financially funded by LPDP (Indonesia Endowment Fund for Education) and accepted as a PhD candidate at Klemens's lab. - Marine Ecology laboratory has given me a remarkable opportunity to experience and explore the ecology of the coastal environment in the temperate zone.

Fieldworks at Schiermonnikoog's mudflat, looking into the interaction, contributions, and roles of shellfish reefs and the algae communities (macro and micro-algae) of the intertidal zone is the core of this thesis. 'Breathtaking' is the right word to summarize the whole process; it is a compilation of long process of designing the experiments, collecting and analyzing samples, surviving in a harsh cold-windy environment, data analyzing, surfing the web, the writing up stage, and last but not least, having a great time with my colleagues. This thesis shows what *Ecology* is about: interaction, balance, acknowledging the power around us and what we have in us.

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**Your assumptions are your windows on the world.
Scrub them off evrey once in a while or the light won't come in.**
-Isaac Asimov-

CHAPTER 1

INTRODUCTION

Rosyta Andriana

Ecological Function

The function of organisms in an ecological system is determined by their interactions with their environment and other organisms. In its simplest form, an ecological function refers to a simple linear cause and effect relation between an organism and an environmental state or another organism, for example an alga depleting nutrients or a predator eating a prey. In a more multifaceted perspective, an ecological function represents a group of organisms inhabiting a larger system, and includes the complex interaction network between all organisms and their abiotic environment (Jax and Setälä 2005). In the larger systems perspective, all the ecological structures and processes that link to the function of a system, can be referred to a system's "functioning". Thus, an ecosystem's functioning is the result of transfer of matter and energy driven by the complex network of different interactions between biotic and abiotic components in the ecosystem (de Groot et al. 2002).

De Groot et al (2000) specified ecological functions into four primary categories: *regulation functions*, regulating the essential ecological processes, such as bio-geochemical cycles and other biospheric processes; *habitat functions*, providing habitat to other organisms as well as promoting the conservation of biological, genetic diversity and evolutionary processes; *production function*, converting energy through photosynthesis, nutrient uptake, creating a source of living biomass; and *information function*, providing an essential reference of cognitive development in relation with human evolution and health.

Considering networks of ecological interactions as system properties and testing the joint function of groups of organisms and species, has moved community ecology toward emergent properties of species assemblages; where the collected function is larger than the sum of all individual functions (Olf et al. 2009). A focus on emergent properties of species assemblages are evident in the ecological literature on biological self-organization, ecodynamics and facilitation theory (Bruno et al. 2003, Ulanowicz 2004, Koppel et al. 2008). For example, Ulanowicz (2004) argued that when ecosystems develop they promote autocatalytic loops that establishes preferred directions of changes that affect networks of interrelated organisms simultaneously. This highlights that the *function* concept to some extent has drifted away from “individual selection” theory. In Darwin’s selectionist approach an organism’s characteristics are created through individual-, not ecosystem-level selection (Wright 1973; Godfrey-Smith 1994; Nunes-Neto et al. 2014). Dussault (2019) argued against the new “group-selection” perspective on function, noting that: (1) the current situation was emphasizing the *context-based* rather than the *history-based* properties of organisms, (2) with the underlining of the processes instead of explaining the presence of organisms within ecosystem, (3) as well as formulating the *use* and *service* functions rather than *design* functions. Hereafter, he asserted that *causal role* theory, significantly supports the functional ecology’s concept by indicating the contributions of organisms to the activities or measurements of the particular ecosystem (Cooper et al. 2016).

Habitat-structuring species

It was in the 2000s that facilitation was reintroduced as an important concept in the ecological literature (Bruno et al. 2003). Since the 1970s, Darwin’s concept of survival of the fittest was largely providing direction for the entire field of ecology. Consequently, a preoccupation with measuring individual fitness and individual adaptation meant that ecology focused almost exclusively on abiotic limitations and negative species interactions; such as competition and predation. Direct facilitation, on

the other hand, is a positive non-trophic interaction between individuals, populations or species. It includes mutualistic processes such as pollination but also relationships where only one species has an obvious net benefit (Olf et al. 2009). As a concept, facilitation was not a new idea, but it is in the past decades that positive interactions have gained momentum as an important part of ecological theory (e.g. Bruno et al. 2003, Maestre et al. 2009, Olf et al. 2009, Bulleri et al. 2011). Today, facilitation is recognized in management as an important consideration to prioritize and even to succeed with active restoration (Silliman et al. 2015, Bulleri et al. 2018).

Already in 1972, the concept of foundation species was introduced to describe the importance of habitat building algae for biodiversity (Dayton 1972). Foundation species are species that provide the fundament for other, associated species by structuring the habitat; i.e. they are habitat-forming species. Since Dayton (1972), coastal ecology has acknowledged the important role of those foundation species that create biogenic substrate and facilitate a wide variety of marine communities; by providing living space, access to resources and refugee from predators and competitors (e.g. Bertness and Callaway 1994, Albert and Reise 1994, Stachowicz 2001, Levenbach 2008, Angelini et al. 2011). Typical and globally distributed marine foundation species form seagrass meadows, shellfish reefs, coral reefs, mangroves and kelp forests; which all facilitate whole communities of organisms by forming or increasing their habitat (Allen and Pye 1992, Altieri et al. 2007, Angelini 2011, Bishop et al. 2013).

Many habitat-forming species not only provide biogenic substrate, they also modify the physical environment; so called ecosystem engineers (sensu Jones et al. 1994). Ecosystem-engineering species facilitate associated communities by mitigating abiotic stressors, improving their performance or providing a niche in an otherwise inhospitable environment (Jones et al. 1997; Wright et al. 2002). One key difference between the habitat-forming and the engineering function of a foundation species, is that the engineering function can manifest on scales much larger than the extent of the biogenic structure (Hastings et al. 2017). Long-range engineering effects can extend for

hundreds of meters (van de Koppel 2015). For example in sedimentary habitats, foundation species such as seagrass, cord grass, and mussels , provide shelter from exposure and thereby modify sediment properties, oxygen conditions, and physical abrasion both on the local scale and across the seascape (Gutierrez et al. 2012, Donadi et al. 2013).

Habitat-forming species also interact with each other, and may create a complex and hierarchical network of positive interactions (Angelini et al., 2011). At local scale, habitat-forming species sometimes create nested clusters, which may lead to facilitation cascades where a basal foundation species functions as a substrate for a secondary foundation species, which in turn facilitates an associated community of organisms (Altieri et al. 2007, Angelini et al. 2011; Bishop et al. 2013; Thomsen et al. 2016; Gribben et al. 2019). Habitat-forming species that also have ecosystem-engineering properties, may form adjacent clusters where they mitigate environmental conditions at a distance, allowing a new habitat-forming species to establish that in turn facilitate other species (Angelini et al. 2011).

Tidal flats

Tidal flats are sediment-dominated systems with gentle slopes that are characterized by highly fluctuating environmental conditions due to the force of ocean tides (Reise 1983, Semeniuk 2005). By periodically being submerged and emerged, tidal flats and their marine communities are exposed to conditions that are distinctly different from the permanently submerged marine habitat: such as air, tidal currents and temperature fluctuations. Tidal flats are therefore characterized by distinct physical properties and biological communities that are adapted to physiological stress (Reise 1983). Across the tidal flat, sediment conditions and biological aspects are determined by exposure periods, hydrodynamic conditions

regulated by tidal-current forces, and ranges in salinity and temperature (Reise 1983, Semeniuk 2005).

Tidal flats have an important function in coastal dynamics, as well as integrated coastal zone management (Davies 1980; Allen and Pye 1992; Flemming B.W. 2005). On the surface, tidal flats are substantially described as un-vegetated areas and fine-grained sediment-rich environments, which stretch out for many kilometers of the coastline in some regions (Davis 1994; Flemming and Hertweck 1994). However, from sea towards land, there is a zonation on the tidal flat: *low tidal flats* are exposed only during low spring tides, and are generally characterized by a gently sloping sandy substrate, with no vegetation on the surface; middle tidal flats are exposed during low neap tides and have a minimal slope; high tidal flats are only flooded during mean and extreme high spring tides and are characterized by muddy/soft sediment substrate that often, but not always, is vegetated (Semeniuk 2005). The biological communities of the unvegetated lower and middle tidal flats are characterized by communities of organisms that live in and on the sediment and are able to move freely as larvae and/or adults (i.e. diatoms, molluscs, polychaetes)(Reise 1983). The biomass of these invertebrate- and algae-dominated communities are often very high, and site-specific community composition and abundances are affected by physicochemical and biological conditions (Semeniuk 2015; Chapman 1977; Adam 1990). Yet, in general, such areas resemble “deserts” of featureless habitat with less aboveground complexity and biodiversity than vegetated habitats (Byers and Grabowski 2014).

The research in this thesis was performed on the tidal flats of the temperate Dutch Wadden Sea. The Dutch Wadden Sea falls into the category of a sandy or muddy tidal flat, with salt marsh, sheltered with barriers in a humid temperate climate (Reineck 1975, Reise 1983). The seemingly homogenous tidal flat in combination with the strong dependence on physically driven external forces that structure the unconsolidated sediment, have led to the dominant view in science, policy and

management that the tidal flats of the Wadden Sea are abiotically driven and dynamic systems (Eriksson et al. 2010). However, the sedimentary character also means that habitat-forming ecosystem-engineering species have an opportunity to significantly modify their own habitats, and thereby improve the living space for themselves and for their associated species (Eriksson et al. 2010, van der Ouderaa, 2021).

Biology of tidal flats in the Wadden Sea

Although the tidal flats of the Wadden Sea may look biologically homogenous, there are at least four distinct community types: flats with muddy sediment, flats with sandy sediment, shellfish beds and seagrass meadows (Reise 1983). Relevant for the discussion of function is that many of the common species that inhabit tidal flats tend to establish aggregations with a high density of individuals, and thereby dominate their respective habitats (Reise 1983). These reef- or bed- forming organisms are ecosystem engineers whose biological activity have distinctly different influences on the dynamics of the sediment they inhabit: some are sediment stabilizers and some are sediment destabilizers (Eriksson et al. 2010). For example, the extensive sandy flats are often dominated by the lugworm (*Arenicola marina*), which is a strong bioturbating species, which destabilizes the sediment. On the higher and more muddy tidal flat, cockles (*Cerastoderma edule*) and dwarf eelgrass (*Zostera noltei*) can form dense beds, which stabilize the shallow sediment by forming a dense matrix right under the sediment surface (Eklöf et al. 2011, Donadi et al. 2013b, 2015). If we move closer to the low water line, blue mussels (*Mytilus edulis*), the tube-forming polychaete worm sand mason (*Lanice conchilega*), and Pacific oysters (*Crassostera gigas* or *Magallana gigas*) form epifaunal reefs that cover the sediment. Since these organisms dominate their respective communities, their interplay with natural sediment dynamics and currents determine the composition of different communities of associated species (Eriksson et al. 2010).

In this thesis, I focus on testing the function of intertidal shellfish reefs on algae communities in the Wadden Sea. Intertidal shellfish reefs are prominent ecosystem engineers with a large ecological function on multiple scales (van de Koppel et al. 2015; see Box 1). Shellfish reefs provide diverse habitats for a rich and biodiverse mix of invertebrates, fish and birds (van der Zee et al. 2012, van de Ouderaa et al. 2021) and thereby they act as biological power stations on the intertidal flat that increase biodiversity, promote primary production and enrich the food web (Christianen et al. 2016, Engel et al. 2017).

In the Wadden Sea, intertidal reefs are nowadays consist of a mix of blue mussels and the invasive Pacific oyster. The Pacific oyster invaded the area three decades ago, and has dramatically changed the composition of the native reefs (Reise et al. 2017b). Today, mussels and oysters mostly live together in mixed beds, so called oyssel reefs (*sensu* Reisse et al. 2017b). If this has changed the function of the reefs significantly is yet unclear. This will be addressed in this thesis.

There is a clear interaction between intertidal shellfish reefs and benthic algae. On the reefs, this is most evident by conspicuous beds of canopy forming bladderwrack (*Fucus vesiculosus* forma *mytili*) that establish on top of the blue mussel aggregations (Albrecht & Reise 1994). The bladderwrack mainly reproduces clonally by means of entanglement of fractured thalli in the byssus of the blue mussels (Albrecht 1998). However, at least as important is the positive effect of shellfish reefs on the microphytobenthos. By mitigating hydrodynamic stress and stabilizing the sediment, the physical structures of the reefs promote microphytobenthos both on the reefs, and up to several 100 meters in their wake (Donadi et al. 2013, Engel et al. 2017). The diatom-dominated microphytobenthos is the most important group of primary producers on the tidal flat in the Wadden Sea (Underwood and Kromkamp 1999, Box 1). It forms a thin layer of photosynthesizing biofilm that covers the sediment and fuel the tidal flat food web (Christianen et al. 2017a).

Box 1. Benthic diatoms on the tidal flat

Benthic diatoms play an important function as primary producers by contributing approximately 50% of total primary production on tidal flats (Underwood and Kramp 1999). They inhabit natural soft-sediments by forming a visible brown biofilm that form a thin layer that covers the sediment. The biofilm is the most important energy source for higher trophic levels in the tidal flat food web (Christianen et al 2017). The biofilm is constructed by secreting extracellular polymeric substances (EPS) which inhibit erosion, stabilize the sediments by increasing sediment cohesion and decreasing bottom roughness (Heip et al 1995, Weerman et al 2010).

Fig. B.1.1 Benthic diatom biofilms, thick brown layers, grow on top of tidal flat's muddy surface at the South-east barrier island Schiermonnikoog, Wadden Sea's Netherlands (Photo credit : R. Andriana).

In general, there are two dominant benthic diatom groups that live in the sediment on intertidal mudflats: epipsammic diatoms that live attached to the sandy sediments, and epipellic diatoms that are motile and live in and on the fine-grain sediment (Trites, et al 2005). The biofilm layer is dominated by epipellic diatoms which have a raphe structure that allow them to move freely and migrate back and forth through the sediment depending on light conditions (Blommaert et al 2018).

As photosynthetic organisms, their distribution and composition in the tidal flat are determined by the environmental factors : the gradients of salinity and nutrient (Underwood et al 1998), light (Blommaert et al 2018), and sediment grain-size distribution as determined by exposure (Colijn and Dijkema 1981, Staats et al 2001). Yet, biological feedback mechanism which involve multiple trophic-level interactions also plays a structuring role in altering the patch-size distribution and vertical distribution of benthic diatoms (Weerman et al 2012, Yin et al 2016).

Box 2. Intertidal shellfish reefs : Blue mussels, Pacific oysters

Intertidal shellfish reef is constructed by reef-forming bivalve species: blue mussel (*Mytilus edulis*) and the invasive Pacific oyster (*Magallana gigas*) (Kochmann et al 2008, Reise et al 2017b). They create biogenic structures on tidal flat, which are known as habitat-structuring species : alleviating hydrodynamic stress in the surroundings, providing shelter to other organisms, which leads to habitat complexity and biodiversity enrichment (Baird et al 2007, van der Zee et al 2015, Engel et al 2017, Eriksson, et al 2017).

Fig. B.2.1 Intertidal shellfish reefs on tidal flat of Schiermonnikoog (South East) during low tide; a dense layer of algae covers the reefs (Photo credit : R. Andriana).

These reef forming organisms alter biotic and abiotic parameters within and their surroundings, up to hundred meters, across the tidal flat (Donadi et al 2013a, 2013b; van der Zee et al 2012; van de Koppel et al 2015; Gutierrez et al 2003) : nutrient hotspots, substrates for other organisms to attach to, and a harbour for larvae and the settlement of juveniles.

The presence of this reef contributes significantly to primary production : the increasement of chlorophyl a concentration, the net production of biofilm and seaweed (Asmus and Asmus, 1991, Baird et al 2007, Engel et al 2017).

Thesis Outline

This thesis investigate the function of shellfish reefs for benthic algae on the tidal mudflat. I investigate effects both on the benthic communities of macroalgae that establish on the reefs, and on the diatoms that form the biofilm in the shallow sediment covering the intertidal.

Table 1. *The important concepts and interpretations of soft-sediment processes' which are addressed during this study (Byers and Grabowski, 2014).*

Concepts	Descriptions
Invasive species	<ul style="list-style-type: none"> - Commonly found in soft sediments; they started their invasion at nearshore habitats, as the starting point as they <i>brought in</i>. - Contributing high impacts due to the characteristics of soft- sediment environments : high disturbances and low advection.
Habitat complexity	Ecosystem engineers have substantial potential in soft-sediments environment as habitat providers, sediment stabilizers or destabilizers and key players of habitat diversity.
Microphytobenthos	<ul style="list-style-type: none"> - Benthic microalgae are fundamental and resilient ecosystem modifiers. - Have an important role in the estuarine food web as food sources.

The results are based on a mix of ecological field studies and field experiments. I performed two field experiments where I created different bioengineering habitats, including blue mussel and Pacific oyster reefs, and studied the effects on macroalgae and on the biofilm (Chapters 2 and 3). I also described sediment conditions, chlorophyll *a* levels and the distribution of diatoms across an intertidal shellfish reef; including different intertidal habitats (Chapters 3 and 4). As a closure of this thesis, I present a preliminary study in identifying benthic diatoms by applying a molecular approach (Chapter 5), which gives a template for future studies in molecular analysis of benthic diatoms.

In my thesis, the following specific questions were addressed:

- Has the transformation of the native blue-mussel reefs by the dramatic increase in population size of the invasive Pacific oyster affected the development of benthic macroalgae on the present shared reefs? (Chapter 2)
- What is the ecological function of shellfish reefs for benthic primary production in the intertidal ecosystem? (Chapter 3)
- How do mussel reefs affect the distribution of marine benthic diatoms? (Chapter 4)
- How to best apply a molecular approach in identifying marine benthic diatoms? (Chapter 5)

In Chapter 2, “A pacific oyster invasion transform shellfish reef structure by changing the development of associated seaweeds”, we tested how the transformation of shellfish reefs by the invasive Pacific oyster shape the marine vegetation on soft bottoms. By creating small-scale reefs of blue mussels or oysters, I demonstrate that blue mussel- and oyster-dominated reefs create a habitat that is suitable for different seaweed communities with very different functioning.

In Chapter 3, “Mussel beds are biological power stations on intertidal flats”, small scale manipulations of reefs and a field study across a mussel bed demonstrated that blue mussel beds increase the concentration of chlorophyll *a* in the sediment, and thus increase the productive biomass of the biofilm, and that production of the biofilm on the tidal flat peaked on the mussel bed.

In Chapter 4, “Intertidal mussel reefs change the composition and size distribution of diatoms in the biofilm”, small scale manipulations of reefs and a field study across a mussel bed demonstrated the large impact of shellfish reefs on biofilm functioning. The reefs changed the community composition of diatoms in the biofilm by promoting higher numbers of smaller sized cells and species with higher photosynthetic rates.

In Chapter 5, “Preliminary study : molecular identification of benthic diatom isolated from the tidal flats of Schiermonnikoog by applying *rbcL* as the genetic marker”, a method test was conducted using a specific gene marker, *rbcL*, on frozen sample of benthic diatoms collected from a tidal flat. Following the procedure of molecular analysis and sequencing by applying BLAST, the results verified high sequence identities along with their phylogenic-likelihood tree.

In chapter 6, I present a synthesis of all chapters and concluding remarks.

@Schiermonikoog (©R. Andriana)

@Intertidal flat of Schiermonnikoog (©I. van der Ouderaa)

We made a living by what we get, but we make a life by what we give.
-Winston Churchill-

CHAPTER 2

A Pacific Oyster invasion transforms shellfish reef structure by changing the development of associated seaweeds

Rosyta Andriana,
Isabelle van der Ouderaa, Britas Klemens Eriksson

ABSTRACT

Biological invasions are reshaping coastal ecosystems across the world. However, understanding the significance of such invasions is often hampered by the lack of process-based research, resulting in a limited mechanistic comprehension of novel ecological interactions and their consequences. The Pacific oyster (*Crassostrea gigas*) has invaded European coasts, resulting in an astonishing transformation of the intertidal shellfish reef communities in the Wadden Sea; from reefs constructed by blue mussels only (*Mytilus edulis*) to mixed reefs dominated by oysters. Shellfish reefs structure the marine vegetation on soft bottoms by accumulating seaweeds. Nevertheless, assessments of the consequences of the oyster take-over have almost exclusively focused on effects on associated fauna. By constructing small-scale reefs dominated by blue mussels or oysters and following the development of seaweeds over summer, we demonstrated that oysters promoted bloom-forming green algae communities with low primary biomass and low habitat complexity. In contrast, blue mussels promoted the development of meadow-like communities dominated by habitat forming brown seaweeds of the genus *Fucus*, with high primary biomass and high habitat complexity. An additional field survey showed that increasing numbers of Pacific oysters on a recently invaded natural blue mussel reef significantly decreased the development of the *Fucus* meadow in spring. Our results indicate that the invasion of oysters may have effects on the structure and function of intertidal reef-communities by changing energy flow and habitat-function.

Estuarine, Coastal and Shelf Science 235 (2020) 106564
<https://doi.org/10.1016/j.ecss.2019.106564>

INTRODUCTION

Intertidal shellfish reefs are biological hotspots that modify their own habitat by constructing biogenic structures (e.g. Baird et al 2007). The physical structures of the reefs mitigate hydrodynamic stress and promote habitat complexity, and thereby increase biodiversity, trophic transfer and food web complexity (van der Zee et al 2013, 2015; Engel et al 2017; Eriksson et al 2017; Christianen et al 2017b). In the North Sea, intertidal shellfish reef systems have undergone dramatic changes in the past decades due to an invasion of the Pacific oyster (*Crassostrea gigas* Thunberg, 1793; alternate representation *Magallana gigas*) that overgrows the dominating native reef forming species; the blue mussel (*Mytilus edulis* L.) (Kochmann et al 2008; Reise et al 2017a, 2017b). Today, the consequences of the oyster invasion for the function of intertidal reefs is uncertain, making current risk assessments ambiguous (Herbert et al 2016; Mortensen et al 2017).

Pacific oysters and blue mussels alter habitat structure in different ways, and therefore influence sediment quality and the composition of associated fauna differently (Kochmann et al 2008; Waser et al 2016). Pacific oysters also facilitate other non-native species (Buschbaum et al 2012). However, there is no confident indication that the Pacific oyster invasion has had negative consequences for the native fauna on the regional scale. In contrast, many invaded blue mussel reefs today have a multi-layered structure, consisting of a mixture of both Pacific oysters and blue mussels, that accommodate a higher biodiversity of associated fauna than comparable pure blue mussel reefs (Markert et al 2010; Reise et al 2017b). The spaces formed between the larger Pacific oysters even provide shelter for blue mussels, protecting them from predation by crabs and overgrowth by barnacles (Kochmann et al 2008; Buschbaum et al 2016; Reise et al 2017b). At the same time, competition for resources between shellfish generate a trade-off, where protection and shelter often come at a cost of slower growth and smaller sizes (Eschweiler and Christensen 2011; Donadi et al 2013a).

By providing a habitat that combines available substrate with shelter from hydrodynamic stress and an increased concentration of nutrients, intertidal shellfish reefs also function as biological power stations that increase the net production of the biofilm, the potential production of plankton and the accumulation of seaweed (Asmus and Asmus 1991; Baird et al 2007; Donadi et al 2013b; Engel et al 2017). However, it is not clear what consequences the current changes in reef structure and function have for primary production. The biofilm is the most important energy source for the intertidal food-web in the Wadden Sea (Christianen et al 2017a), contributing strongly to trophic transfer. However, the blue mussel reef system is characterised by seaweed, which is a mix of brown, green and red macroalgae that dominate the primary producer biomass on most reefs (Baird et al 2007). Outside the reefs, macroalgae are found in relatively low abundances due to the lack of hard substratum to attach to. The most conspicuous reef associated seaweed community consists of a habitat forming brown algae, *Fucus vesiculosus* f. *mytili* (Nienburg) Mathieson and Dawes (2017) (from now on *Fucus*), that forms thick meadows of ca 0.5 m long thalli that cover blue mussel reefs on intertidal flats. The thalli are attached by entanglement in the byssus threads that the mussels produce to attach to each other (Albrecht and Reise 1994; Albrecht 1998). The *Fucus* cover develops on the mussel reefs in early spring, mature over the summer and usually disappears again during the winter. Oysters do not produce byssus threads, raising the concern that when oysters overgrow the native blue mussels, the *Fucus* communities will be replaced by fast growing, much less complex green algae, that quickly form mats on hard substrates and exclude other algae by pre-empting space for settlement (Eriksson et al 2007; Nehls and Büttger 2007). In this study we addressed the potential effects of the oyster invasion on seaweed meadows, by testing the development of macroalgae on different types of small-scale bivalve aggregations created on the intertidal.

MATERIAL AND METHODS

Bio-manipulation experiment

This study was conducted on an intertidal mudflat south of the island Schiermonnikoog (Dutch Wadden Sea, N 53.466°, E 6.187°). The mudflat is ca. 2 km wide at low tide and structured by reef-forming bivalve species: the invasive Pacific oyster (*Crassostrea gigas* Thunberg 1793; alternate representation *Magallana gigas*) and the blue mussel (*Mytilus edulis* L.), which form elevated reefs, and the common cockle (*Cerastoderma edule* L.), which forms submerged banks. These species alter the system by constructing physical structures for associated organisms (Donadi et al 2015; Eriksson et al 2017).

We designed a bio-manipulation (BM) experiment to investigate the facilitative effect of different shellfish reef forming species on the development of seaweed. The experiment was set up on the exposed and the sheltered side of a large mixed shellfish reef located ca. 500 m parallel to the shore and consisting of blue mussels, Pacific oysters and common cockles. The exposed site was 100 m seaward of the shellfish reef close to a gully and characterized by sandy sediment with low organic content and high erosion (organic matter content 0–1 cm: $0.90 \pm 0.15\%$ weight loss on ignition; organic matter content 0–5 cm: $0.82 \pm 0.14\%$ weight loss on ignition; plaster erosion: $23.0 \pm 1.2\%$ loss per tide; mean $\pm$ SD). The sheltered site was 300 m leeward of the shellfish reef and characterised by muddy sediment with high organic content and low erosion (organic matter content 0–1 cm: $1.69 \pm 0.55\%$ weight loss on ignition; organic matter content 0–5 cm: $1.76 \pm 0.28\%$ weight loss on ignition; plaster erosion: $15.0 \pm 2.9\%$ plaster loss per tide; mean $\pm$ SD) (see sampling methods below). The bio-manipulation treatments consisted of adding cockles, blue mussels and Pacific oysters to experimental plots with an area of 0.25 m², in four combinations: (i) no addition of bivalves (no addition control - C); (ii) addition of 250 alive cockles (artificial cockle bank - CA); (iii) adding 200 alive blue mussels (artificial blue mussel reef - M); (iv) adding 25 alive Pacific oysters and 50 alive blue mussels (artificial oyster dominated

reef - O). The oyster dominated reef treatment corresponded to ca 60% cover of oysters and 20% cover of blue mussels in the plots (Pacific oysters were ca. five times bigger than the blue mussels). All bivalves were collected *in situ* on location and distributed evenly over the surface of the plots (Fig. 1). Only individuals free from overgrowth of barnacles or seaweed were used. The blue mussel and oyster reef treatments mimicked the natural variation across the shellfish reef, ranging from blue mussel only to oyster dominated patches, while the cockle treatment mimicked cockle bank densities on the sheltered side of the reef. For the first 24 h of the experiment, all plots were bordered by a 1 dm high metal fence (chicken gauze; mesh size 1 cm) to contain the bivalves until they stabilised. For the rest of the experiment a rope was struck between corner poles, to avoid birds preying on the added bivalves. Three replicates of each treatment were randomly assigned to a block of 12 plots in total at each site (exposed and sheltered), resulting in 24 plots in total. These plots were set up in three rows perpendicular to tidal currents. The distance between plots was 3 m on each side (Fig. 1).

The experiment was set up in the end of June (June 22, 2017) and terminated in October (October 18, 2017). Sampling occurred 2, 4, 8, and 14 weeks after the start of the experiment. During this time we documented the development of seaweed in the plots as the percentage of macroalgae coverage, determined to species. We also counted the number of bivalves in each plot (to the species level) and took sediment samples for measurement of sediment organic matter content. The organic matter samples were collected with a plastic syringe (diameter: 2.6 cm) at two different depths, 1 and 5 cm. The samples were placed into pre-labelled plastic bags, stored on ice and put in a -20 °C freezer until further processing. Organic matter content was analysed by drying the sediment for 48 h at 65 °C and then determined by the weight loss on ignition after burning at 550 °C for 4 h. Hydrodynamic erosion was measured for each site using plaster poles (see plaster erosion method in Donadi et al 2013b). Cockle density in the cockle treatments was estimated by taking a small core subsample (1 dm²) from the no-addition and cockle addition plots after 4 weeks. After counting the cockles, the sediment and cockles were placed back into the plots.

It is clear, that compared to a natural reef, the size of the experimental plots were small and the experiment did not fully represent the many scale dependent effects that we earlier have documented on natural reefs (e.g. Donadi et al 2013a, 2013b). To be able to translate what the experimental results mean on natural scale we therefore performed a complementary field study.

Field study

To verify that the small scale experimental results are relevant also on a natural reef scale, we studied the accumulation of *Fucus* after the winter on two natural mixed shellfish reefs close to the experimental site, one dominated by blue mussels ('the blue mussel reef') and one dominated by Pacific oysters ('the Pacific oyster reef'). The blue mussel reef was only recently invaded by the Pacific oyster. The reef is still dominated by blue mussels, but the past five years oysters have increased to up to 50% cover in some patches. The Pacific oyster dominated reef harbours a mix of Pacific oysters, cockles and blue mussels. *Fucus* dominates the seaweed cover on the reefs, but it normally completely disappears in winter, probably due to storms and scour (pers. obs.). After the winter, *Fucus* quickly regenerates to form a thick meadow that covers the reef again. We visited the reefs in early spring (March 6, 2019), just after the *Fucus* cover had emerged and started to grow, to document the development of seaweed depending on the composition of the natural reef substrate approximating the conditions of the experiment. We placed 16 0.25 m² squares in the newly developed *Fucus* meadows across each reef at lower elevations (since *Fucus* do not develop on the oyster dominated ridges). The plots were placed to represent the full gradient of *Fucus* cover on each reef, avoiding areas devoid of *Fucus* and bare spaces; meaning that we can estimate the impacts of mussels and oysters on seaweed development on each reef, but not compare the covers between reefs. First we estimated the cover of *Fucus* and carefully removed the thalli; and then we counted the number of blue mussels and Pacific oysters in each plot.

Fig. 1. Bio-manipulation treatments: experimental reefs created by not adding any bivalves (no addition treatment), adding 250 cockles (cockle bank treatment), adding 200 blue mussels (mussel reef treatment) and adding 25 Pacific oysters and 50 blue mussels (oyster reef treatment).

Data analysis

The effect of the bio-manipulation on the accumulation of seaweed was analysed with general linear mixed models (GLMM) using the *nlme* package in R (version 3.1–140), crossing the independent factors bio-manipulation (no addition control, cockle addition, blue mussel addition and oyster addition) and the repeated measure experimental time (2, 4, 8 and 14 weeks). To account for the repeated measure, we included a temporal autocorrelation structure for each plot (random factor) across experimental time. We were particularly interested in if the statistical interaction between the experimental treatments and time was different between the two sites. We therefore analysed each site separately. Transformation of the data in general deteriorated a good fit to the normal distribution and homogenous variances, and was therefore not considered necessary. To distinguish differences between treatment combinations, Tukey's HSD post-hoc tests was performed. We evaluated the stability over time of the reef treatments by fitting similar GLMM's to the number of blue mussels in the blue mussel and oyster addition treatments (crossing experimental manipulation and time), and the number of oysters in the oyster addition treatment

(testing the trend over time only). Counts were square root transformed. Stability of the cockle treatment was analysed by comparing cockle numbers between the small core-subsamples in the no-addition and cockle addition plots, using a GLM with site as a fixed factor.

The impact of the oyster invasion on the development of *Fucus* cover on natural reefs was analysed by simple linear regressions after visual inspection of distributions. We tested the relation between the number of blue mussels and Pacific oysters on the development of *Fucus* cover on the mussel and oyster dominated reefs separately, because of systematic differences in the cover of *Fucus* between the reefs. In the analyses we only included alive (closed) blue mussels, since these are the ones that produce byssus threads; in practice this meant that we did not include open and separated shell material ($\ll 5\%$ of the mussel matrix). For oysters, however, we included both dead and alive oysters in the analyses because dead individuals make up a large and natural part of the oyster matrix (34% of the individuals), and also dead oyster shells take up space from the blue mussels.

RESULTS

Bio-manipulation experiment

The bio-manipulations created small-scale bivalve habitats that were stable for about 3 months of experimental time: including blue mussel reefs (mussel addition), Pacific oyster dominated reefs mixed with blue mussels (oyster addition) and cockle banks (cockle addition) (Appendix A, Fig. 2). At the exposed site, both blue mussels and oysters decreased gradually in their respective reef treatments (main effect of time; blue mussels: $F_{1,13} = 85.4$, $p < 0.001$; oysters: $F_{1,5} = 207.45$, $p < 0.001$; Fig. 2a and b). After a strong storm in August (experimental week 12) all blue mussels and oysters disappeared from the exposed site. At the sheltered site, the oyster reef treatments stayed stable over summer until September, when the number of alive oysters decreased

significantly, leaving a matrix of alive oysters mixed with dead oyster shells (main effect of time; alive oysters: $F_{1,5}=20.83$, $p < 0.001$; Fig. 2c). The mussel reef treatment created small aggregations over the summer at the sheltered site, with 10 times the abundance of blue mussels compared to all other treatments (Fig. 2d). However, after the storm in August, the mussel reef treatment disintegrated and all blue mussels were washed away, except in the oyster reef treatment. In the oyster reef treatment the blue mussels were protected from the storm and more than doubled in abundance between July and September (Table A1, significant interaction effect between time and biomanipulation; Fig. 2d).

Over the course of the experiment, distinctly different seaweed communities developed in the different bio-manipulation treatments; the artificial mussel reefs facilitated thick brown algae meadows of *Fucus*; the artificial oyster reefs facilitated bloom forming green algal mats dominated by the genus *Ulva*; and the artificial cockle beds did not facilitate seaweed cover at all (Fig. 3). At the exposed site, the cover of brown algae was very low in all treatments, except for a short peak of 20% cover on the oyster dominated reefs after 2 weeks (Table 1, no significant treatment effects; Fig. 3a–d). However, at the sheltered site, the development of *Fucus* followed the trends in blue mussel abundances. Here, the mussel reef treatments quickly developed a thick meadow of entangled *Fucus* with 3–4 dm long thalli that stayed above 60% cover; until the reefs were washed away during the storm in August (Table 1, significant interaction between time and the bio-manipulation treatment; Tukey's HSD post-hoc test, $p < 0.001$: mussel addition > oyster addition = cockle addition = no addition treatment; Fig. 3e–h). In the oyster reef treatment, *Fucus* started to accumulate in late summer, together with the documented increase of mussels inside the oyster matrix, reaching 30% cover in September. The oyster reef treatments were otherwise characterised by mats of green algae, with a peak of ca. 40% cover from the middle of June to July at both the exposed and sheltered sites (Table 1, significant interaction effect between time and biomanipulation; Tukey's HSD post-hoc test, $p < 0.001$: oyster addition > mussel addition = cockle addition = no addition treatment; Fig. 3). Red algae of the genus

Gracilaria showed a small peak in the mussel reef treatments after 2 weeks at the sheltered site, but in general occurred very sparsely in all treatments during this study (Table 1, no significant treatment effects; Fig. 3).

Field study

Blue mussels strongly promoted *Fucus* cover and the invasion of the Pacific oyster on the mussel dominated reef significantly decreased the development of *Fucus* (Fig. 4). The plots on the mussel dominated reef had on average 2.5 times as many blue mussels and half of the number of Pacific oysters as the oyster dominated reef (mussel reef: # blue mussels = 254 ± 91 , # Pacific oysters = 80 ± 50 ; oyster reef: # blue mussels = 105 ± 54 , # Pacific oysters = 152 ± 29 ; mean # $\text{m}^{-2} \pm \text{SD}$). *Fucus* increased strongly with the number of alive mussels both on the mussel reef (linear model: $n = 17$, $r = 0.895$, $p < 0.001$) and the oyster reef (linear model: $n = 16$, $r = 0.891$, $p < 0.001$; Fig. 4a). There was a strong negative correlation between oysters and mussel abundances on the reefs (Pearson product-moment correlation: $n = 33$, $c = -0.873$, $p < 0.001$). Accordingly, *Fucus* cover decreased with increasing numbers of Pacific oysters (alive and dead shells) both on the mussel reef (linear model: $n = 17$, $r = -0.888$, $p < 0.001$) and the oyster reef (linear model: $n = 16$, $r = -0.696$, $p < 0.01$; Fig. 4b). For every extra oyster shell found in the plots on the mussel reef, the cover of *Fucus* decreased with 2.0% (Fig. 4b).

Fig. 2. The abundance of Pacific oysters (a & c) and blue mussels (b & d) in the different experimental reefs at an exposed (a & b) and a sheltered (c & d) site. Time shows the week from the start of the experiment in the beginning of July. The experimental reefs were created by not adding any bivalves (no addition treatment: dotted line, open squares), adding 250 cockles (cockle bank treatment: solid green line, filled triangles), adding 200 blue mussels (mussel reef treatment: solid blue line, filled circles) and adding 25 Pacific oysters and 50 blue mussels (oyster reef treatment: striped red line, filled diamonds). Error bars show standard errors of the mean values.

DISCUSSION

Different algal communities developed on the different small-scale shellfish reefs that we constructed on the intertidal mudflat. On the reefs consisting of native blue mussels, a *Fucus*-dominated meadow community quickly developed that remained throughout the summer. On the Pacific oyster dominated reefs, mats of bloom-forming green algae established instead, and the cover of *Fucus* stayed low throughout the summer. In addition, the invasion of Pacific oysters decreased the development of the *Fucus* meadow after the winter on a previously pure blue mussel dominated reef. This demonstrates that the invasion of Pacific oysters may have significant effects on the structure and function of intertidal reef communities. While the green algae dominated reef communities have low primary biomass and low habitat complexity, brown algae such as *Fucus* are important foundation species that promote an array of associated organisms by increasing habitat complexity (Dayton 1975; Albrecht and Reise 1994; Jenkins et al 1999a). The contribution of fucoid communities to marine ecosystem functioning is well documented and includes; modification of consumer and resource control, nutrient effects on net primary production and biodiversity, predation effects and fish abundance, and increases in sediment accumulation (Albrecht 1998; Eriksson et al 2006a, 2006b; 2007, 2009). On the island of Sylt, Germany, the cover of *Fucus* decreased dramatically on blue mussel reefs in the early 2000s, leading to a shift in community structure (Buttger et al 2008). The reefs were recently invaded by Pacific oysters, but the cause of the decline in *Fucus* was not established. Our results provide a mechanistic link that suggest that there may be a direct connection between the invasion of the Pacific oyster and declines in *Fucus* meadows.

The remarkably fast development of primary biomass in our experiment highlights the ecological importance of byssus threads in intertidal ecosystems. The *Fucus* community developed a complete cover of fully grown 3–4 dm long thalli on the artificial mussel reefs in just 2 weeks by means of entanglement in the byssus threads of the blue mussels. Entanglement of clonal fragments is a common reproductive

strategy among seaweeds (Eriksson and Johansson 2005; Hurd et al 2014). However, the importance of fragmentation for building the habitat in our system, where entanglement is the base of a structurally complex kelp community that dominates primary biomass (Albrecht and Reise 1994; Baird et al 2004), may be unprecedented in marine ecosystems. This strongly suggests that the ecological consequences brought by the large-scale oyster invasion of intertidal shores are not restricted to direct effects on competing bivalves and associated invertebrates, but may have cascading implications on an ecosystem level, due to fundamental differences in basic ecological functions of blue mussels and oysters.

In general, both the function and stability of the experimental reefs dominated by the invasive oyster were more resilient to hydrodynamic stress than the reefs constructed only with native blue mussels. The ability of the blue mussel reef treatments to attach and accumulate *Fucus* thalli degenerated drastically over time at the exposed site. Exposure has a large impact on byssus production and behavior (Van Winkle 1970); at high flow rates byssus production decreases (Moeser et al 2006; Carrington et al 2008) and blue mussels actively choose to attach to different substrates depending on perturbation rates (Kangeri et al 2014). The blue mussels might even have avoided attaching to *Fucus* thalli at high exposure due to the risk of being pulled away by the additional drag. In contrast, the experimental oyster reefs performed similarly at the different levels of exposure: throughout the summer, the numbers of oysters and green algal development were comparable at the exposed and the sheltered site.

Our results also support that novel and complex interactions between the invading oysters and the native blue mussels currently determine the resilience of these intertidal reef communities (Eschweiler and Christensen 2011; Buschbaum et al 2016; Reise et al 2017b). As filter feeding ecosystem engineers that share a food source and optimal feeding areas, blue mussels and oysters compete for food (Reise et al 2017b). However, the large biogenic structure produced by the oysters also provides significant shelter for the smaller blue mussels. Accordingly, blue mussels are often found thriving deep in the oyster matrix where they are protected both from predation by crabs

(Eschweiler and Christensen 2011; Waser et al 2015) and overgrowth by barnacles (Buschbaum et al 2016). Here we show that the oyster matrix also protects mussels from physical disturbance from hydrodynamic stress. In August, a storm wiped out all experimental blue mussel reefs, while the blue mussels thrived and even doubled in numbers when protected by the oyster matrix in the experimental oyster reefs.

Fig. 3. The development of brown (blue solid lines, circles), red (red dotted lines, diamonds) and green (green striped lines, triangles) macroalgae on different experimental reefs at an exposed (a–d) and a sheltered (e–h) site. Time shows week from the start of the experiment in the beginning of July. The experimental reefs were created by not adding any bivalves (no addition treatment: a & e), adding 250 cockles (cockle bank treatment: b & f), adding 200 blue mussels (mussel reef treatment: c & g) and adding 25 Pacific oysters and 50 blue mussels (oyster reef treatment: d & h). Error bars show standard deviations of the mean values.

The invasion of the Pacific oyster has created a novel entity of mixed oyster-mussel reefs that today dominate the intertidal reefs in the Wadden Sea; so-called “oyssel” reefs (Reise et al 2017b). It has been argued that the oyster-mussel reefs have replaced the ecological function of pure blue mussel beds (Markert et al 2010) and that these “oyssel” reefs will be more persistent to physical disruption, because oysters are larger and anchor themselves deeper in the sediment than blue mussels (Reise et al 2017a, 2017b). Here we provide **the first experimental evidence that mixed oyster reefs are more resilient to perturbation than pure blue mussel reefs**. However, we also **demonstrate that the invasion of oysters in the short term promote a transformation of furoid meadows to green algae grasslands, with magnitudes lower primary biomass and habitat complexity**. This change is driven by a fundamental mechanistic difference between blue mussels and oysters, where the decrease in blue mussels leads to less presence of byssus threads that attach the furoids to the reefs. **Marine meadows of furoids, kelps or seagrass are all highly productive communities with significant ecosystem functions and services for associated organisms across the food web; highlighting the need for ecosystem-based research that includes a mechanistic understanding of ecological processes to document the effects of invasive species.**

Fig. 4. The relation between percentage cover of the brown algae *Fucus vesiculosus* and the number of a) blue mussels and b) Pacific oysters on a blue mussel dominated shellfish reef (solid blue line, filled blue circles) and on a Pacific oysters dominated shellfish reef (dotted red line, open red circles). The lines (both dotted and solid) show statistically significant linear relations ($p < 0.01$).

Table 1 Results of GLMM testing the effect of shellfish reef treatments (adding mussels, adding oysters, adding cockles and no addition control), and time (five levels); on different algal communities (brown, green and red algae) at an exposed and a sheltered site. Time was included as a repeated measurement.

	df	brown algae (<i>Fucus sp.</i>)		green algae (<i>Ulva sp.</i>)		red algae (<i>Gracilaria sp.</i>)	
		F	p	F	p	F	p
<i>Exposed site</i>							
Biomanipulation treatment (Bio)	3.8	2.33	0.151	16.69	<0.001	3.556	0.067
Time (T)	1.44	2.6	0.111	0.76	0.388	1.25	0.269
T x Bio	3.44	1.33	0.277	0.51	0.679	0.93	0.434
<i>Sheltered site</i>							
Biomanipulation treatment (Bio)	3.8	16.29	<0.001	11.52	0.003	1.14	0.391
Time (T)	1.44	2.55	0.1173	1.71	0.198	4.23	0.045
T x Bio	3.44	10.77	<0.001	0.22	0.879	1.76	0.169

ACKNOWLEDGEMENTS

We thank Vereniging Natuurmonumenten and the Province of Friesland for granting us permission for field work on the tidal flats and special thanks to the 2017 Marine Research master students Jorn Claassen and Danielle Megan Crowley, and the volunteers Ivana Stavasius, Vicki Venizilos and Cecile Deelman-Driessen, for helping us setting up and performing the field experiment. We also thank two anonymous reviewers for valuable comments on the first version of the manuscript. This work was supported through a scholarship of the Indonesia Endowment Fund for Education (LPDP) to R. Andriana.

Appendix A1. Results of the bio-manipulation experiment on bivalves

The addition of reef building bivalves to the plots on the mudflats generated distinct small-scale reefs that were relatively stable over the course of the experiment: blue mussel reefs (mussel addition) and Pacific oyster dominated reefs mixed with blue mussels (oyster addition). Pacific oysters only occurred in the oyster reef treatment. Blue mussel numbers were significantly higher in the mussel compared to the oyster reef treatments (Table S1, significant main effects of biomanipulation; Tukey's HSD post-hoc test, $p < 0.01$: mussel addition $>$ oyster addition). Four weeks into the experiment, cockle abundances were still significantly higher in the cockle bank treatment (exposed site: 855 ± 270 ; sheltered site: 1327 ± 668 ; mean $m^{-2} \pm SD$), compared to the no addition treatment (exposed site: 0 ± 0 ; sheltered site: 707 ± 153 ; mean $m^{-2} \pm SD$) (significant main effect of cockle additions: $F_{1,9} = 18.7$, $p = 0.002$).

Appendix A1 (continued)

Table A1 Results of GLMM testing the effect of shellfish reef treatments (adding mussels, adding oysters and mussels), and time (four levels – week 8 cancelled from the analyses due to missing data); on the number of blue mussels (*Mytilus edulis*) at an exposed and a sheltered site. Time was included as a repeated measurement.

	Exposed site			Sheltered site		
	df	F	p	df	F	p
Bio-manipulation treatment (Bio)	1.4	20.87	0.010	1.4	10.66	0.031
Time (T)	1.13	85.43	<0.001	1.12	5.69	0.034
Bio x T	1.13	2.92	0.111	1.12	24.86	<0.001

#Sunrise at Intertidal flat of Sciermonnikoog (©R. Andriana)

**Instead of worrying about what you cannot control,
shift your energy to what you can create.**
-Roy T. Bennet-

CHAPTER 3

Mussel beds are biological power station on intertidal flats

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ABSTRACT

Intertidal flats are highly productive areas that support large numbers of invertebrates, fish, and birds. Benthic diatoms are essential for the function of tidal flats. They fuel the benthic food web by forming a thin photosynthesizing compartment in the top-layer of the sediment that stretches over the vast sediment flats during low tide. However, the abundance and function of the diatom film is not homogeneously distributed. Recently, we have realized the importance of bivalve reefs for structuring intertidal ecosystems; by creating structures on the intertidal flats they provide habitat, reduce hydrodynamic stress and modify the surrounding sediment conditions, which promote the abundance of associated organisms. Accordingly, field studies show that high chlorophyll a concentration in the sediment co-vary with the presence of mussel beds. Here we present conclusive evidence by a manipulative experiment that mussels increase the local biomass of benthic microalgae; and relate this to increasing biomass of microalgae as well as productivity of the biofilm across a nearby mussel bed. Our results show that the ecosystem engineering properties of mussel beds transform them into hot spots for primary production on tidal flats, highlighting the importance of biological control of sedimentary systems.

Estuarine, Coastal and Shelf Science 191 (2017) 21-27

<http://dx.doi.org/10.1016/j.ecss.2017.04.003>

INTRODUCTION

Benthic microalgae are important primary producers in intertidal soft-sediment habitats where they contribute up to 50% of total primary production (Underwood and Kromkamp 1999). In these highly productive areas that have a great ecological and economical value across the globe (Heip et al 1995), benthic microalgae fuel the benthic food web by forming extensive biofilms that support a vast array of organisms (Decho 2000; Stal 2003; Kromkamp et al 2006; Markert et al 2013; Rigolet et al 2014). Resource availability and grazing play important roles in regulating benthic microalgae (Underwood and Kromkamp 1999; Weerman et al 2011a, 2011b). However, on tidal flats, large-scale heterogeneity in the abundance and productivity of benthic microalgae is commonly attributed to abiotic conditions, where increasing hydrodynamic stress decrease benthic microalgae biomass by resuspension of the sediment (de Jonge and van Beusekom 1995; van der Wal et al 2010). Recently, we have recognized the importance of biological control over local hydrodynamic conditions on intertidal flats (van der Zee et al 2012; Donadi et al 2013a) and shown that the high abundances of benthic microalgae correlate strongly with the occurrence of mussel beds (Donadi et al 2013b; Nieuwhof et al 2016 personal communication).

Organisms that modify their habitats can facilitate complex food-webs by providing structural complexity and improving environmental conditions for many organisms (Olff et al 2009; Kéfi et al 2015; van der Zee et al 2016). On tidal flats, above-ground aggregations of bivalves such as mussels or oysters can build extensive habitat-forming reefs (e.g. mussel beds). These structures are of fundamental importance for biological control of ecosystem structure and properties (Commito et al 2008; Gutiérrez et al., 2011; van der Zee et al., 2012; Donadi et al., 2013a, 2015). By creating large emergent structures in the otherwise predominantly flat and soft-bottomed landscape, bivalve reefs generate habitat for many other species that live in or on the sediment (van der Zee et al 2012; Nieuwhof et al 2015). The reefs physically protect the surface sediment against erosion and resuspension, and furthermore increase organic matter

content via suspension feeding and biodeposition (Widdows and Brinsley 2002). The habitat modifying properties and ecosystem effects extend up to several hundred meters around reefs (van der Zee et al 2012; Donadi et al 2013a, 2013b, 2015; van de Koppel et al 2015), which is reflected by a conspicuous increase of benthic microalgae biomass in the vicinity of intertidal mussel beds (Donadi et al 2013b). Due to these impacts on a large spatial scale, it can be assumed that the interaction between benthic microalgae and intertidal bivalve reefs contribute significantly to coastal production. However, the assumed regulatory importance of bivalve reefs for microalgae biomass is based on observational data and statistical modelling only, while the causal link of (living) bivalves facilitating benthic diatoms have not been extensively examined. Consequently, we lack conclusive empirical evidence of the facilitation effect. In addition, due to limited measurements of actual productivity, we have a poor understanding of how the increased biomass of microalgae (commonly estimated by chlorophyll a concentration) around reefs relate to productivity of the system.

In this study, we tested the hypothesis that mussel beds increase the local biomass of benthic microalgae on a tidal flat. First, we showed that the biomass of benthic microalgae was consistently elevated across a mussel bed over several years and related this to higher primary productivity. We then used empirical evidence from a small-scale field experiment to demonstrate that the addition of live mussels to bare plots facilitates benthic microalgae.

MATERIALS AND METHODS

Set-up transects

We set up two parallel transects spanning a distance of 1 km each on a tidal flat south of the island Schiermonnikoog (latitude 53.47°N, longitude 6.23°E, Friesland, The Netherlands; Fig. A.1a-b; Table A.1). This tidal flat is a mudflat with varying sediment grain types ranging from fine mud to sand. During low tide, the flat falls completely dry and the tidal range is about 3.5 m. The two transects were 300 m apart and perpendicular to the coast. One transect crossed a *Mytilus edulis* (blue mussel) reef that was ca. 100 m wide and extended for approximately 250 m along the coast; the other one was in a habitat without mussels present. The mussel bed is elevated and exhibits spatial self-organization on two scales: (1) a banded pattern with mussels on top of several meter large hummocks of accumulated sediment and small pools of 1-2 m in diameter, that are void of mussels and retain water during low tide (Liu et al 2012), and (2) a labyrinth-like banded pattern of small mussel clusters that aggregate on the 5-10 cm scale (van de Koppel et al 2008), but that changes into a thick homogenous cover of mussels at peak densities on the hummocks. We established the first point of each transect 350 m coastward of the mussel bed (about 500 m from the shore) and placed subsequent points every 50 m in seaward direction up to 100 m behind the mussel bed (last point ca. 1000 m from the shore). The transect points were selected to cover a visible plume of muddy sediment that extended around the mussel bed.

In June 2012, we sampled chlorophyll *a* concentration at six transect points in both transects (-300 m, -200 m, -100 m, 0 m, +100 m, +150 m distance to the mussel bed/the corresponding tidal elevation in the no mussel bed habitat, where negative values mean distances coastward of the mussel bed/the corresponding tidal elevation in the no mussel bed habitat and positive distances seaward of the mussel bed/the corresponding tidal elevation in the no mussel bed habitat). Distance to the mussel bed is hereafter referred to collectively as distance to the mussel bed in both habitats.

Sampling was replicated spatially, by including samples 50 m to the right and 50 m to the left of each transect point ($N = 36$). In 2015-16, we took chlorophyll a and organic matter samples at five similar transect points in both transects (-300 m, -200 m, -100 m, 0 m, +100 m, +150 m distance to the mussel bed, where negative distances are coastward and positive distances are seaward of the mussel bed), but instead of two spatial replicates we repeated the sampling six times in total (October 2015, October 2016, April 19, 2016, April 29, 2016, May 2016, June 2016). Due to unexpected weather conditions, we could not sample the two last transect points for the no mussel bed habitat in October 2015 (0 m, +100 m) and had to abandon the last sampling point (+100 m) in both habitats in April 2016 ($N = 56$).

We measured photosynthetic yield of the sediment as proxy for benthic microalgae productivity at two different time points. In June 2012 and June 2016, we took triplicate samples in five transect points per habitat.

Set-up field experiment

We designed an experiment to analyze the local effects of mussel presence on benthic microalgae in small-scale plots of 0.5m^2 (Fig. A.2a). Thus, we did not simulate the hierarchical spatial structure of intertidal mussel beds (as described above; see also Snover and Commito, 1998; Kostylev and Erlandsson, 2001; Commito et al., 2006), or their long-range effects (Donadi et al., 2013a, 2013b; van de Koppel et al., 2015). Our experiment allowed us to assess the effect of mussels at the plot scale, avoiding possible confounding effects that different environmental factors could have when including multiple spatial scales in the experimental design (Wiens 1989; Commito et al 2006).

We set up the mussel facilitation experiment in three different sites on the same tidal flat as the transects, south of Schiermonnikoog island (Fig. A.1a, c). The three sites were placed at the same tidal elevation, meaning that all plots fell dry at the same time during low tide, but along a gradient of influence by mussel beds which also means that they differ in sediment erosion, organic matter content and infauna community

composition (van der Zee et al 2012; Donadi et al 2013b, 2015). Site 1 was placed in the transect without a mussel bed; Site 2 coastward of the mussel bed included in the mussel bed transect (300 m to the east of Site 1); and Site 3 coastward of another larger mussel bed that is 100e200mwide and extends almost 1000m along the coast (2000 m east of Site 1; Fig. A.1a).

In each site, we tested the effects of adding mussels on benthic microalgae biomass. For this, we prepared four different treatments with three replicates in each site, leading to 36 experimental units (plots) in total. Each individual plot had an area of 0.25 m² (plot dimension: 0.5 m by 0.5 m) and each corner of the plot area was marked with a plastic pole. The poles were 66 cm long and inserted about 30 cm deep into the sediment. The distance between the plots was 5 m on each side.

The experiment combined two mussel addition treatments and two controls in a factorial design with: a fenced control (FC; Fig. A.2b), a fenced mussel addition treatment (FM; Fig. A.2c), a semi-caged control (CC; Fig. A.2d), and a semi-caged mussel addition treatment (CM; Fig. A.2e). For the two mussel addition treatments (FM, CM), we collected live *Mytilus edulis* and distributed them evenly in the plots so that the surface of the entire plot was covered. After the addition, the mussels organized themselves in the plots overnight by creating a spatial pattern of 5-10 cm banded aggregations (Fig. A.2e). Placing experiments on an intertidal flat may lead to critical artifacts because of changes to water flow caused by equipment rather than treatments, but also because of changed predation rates since the experiment may hinder or attract natural predators. This is critical when placing bivalves on the tidal flat, since they become islands of food for both birds and crabs that quickly consume the treatments. We therefore constructed two types of experimental controls: one semi-caged control (CC) that excluded both crab and bird predation but may have strong effects on flow attenuation; and one fenced control that only excluded bird predation but with minimal effects on flow. The semi-caged control consisted of a coarse plastic coated metal net that was wrapped around the marking poles. The net (mesh size: 1.2 cm) was placed

directly on the seafloor with no space below it and had a height of 25 cm. The fenced control (FC) had a string attached to the poles at about 25 cm height, wrapping around the plots as protection against predation from birds (van Gils et al., 2012). The distribution of the plots was randomized within sites. Deployment of all plots disturbed the sediment in the same way, as all treatments had the same basic built with the four plastic poles in each corner. The experimental plots were set-up in three rows perpendicular to the tidal currents; the most seaward row of treatments was hit by the tides first in all three sites (Fig. A.1a,c, Fig. A.2a).

To test for experimental artifacts, we also included a control without a fence that was only marked in the corners of the plot (no string between the poles; randomized among the other treatments at each site; $N = 9$; Appendix B).

Deployment of the experimental plots took place in the end of April 2015. After more than a month, in June 2015, we took chlorophyll *a* and organic matter samples in all experimental plots. We also collected data on hydrological conditions by using dissolution plasters as proxy for hydrodynamic stress (for methods see Donadi et al., 2013b). The dissolution plasters are cylindrical plaster molds which we expose to the hydrodynamic conditions of the study area during high tide and then estimate erosion by calculating the relative plaster weight loss. We deployed two dissolution plasters in each experimental plot for two tidal cycles before the final sampling.

Due to a severe storm in the end of May 2015, we lost three replicates of the semi- caged control (CC; two in Site 1 and one in Site 3), so that the total $N = 6$ for this treatment.

Sampling and analysis

Chlorophyll *a* concentration in the sediment was measured as proxy for benthic microalgae biomass. For the chlorophyll *a* samples we collected three cores (diameter: 26 mm, depth: 2 mm) in the respective transect points. We pooled the sediment from all three cores and wrapped them onto a 10 x 10 cm piece of aluminum foil to prevent exposure to light. The sediment in the foil was placed into small labeled plastic bags which were sealed and stored on ice in the dark immediately after collection. The samples were transported in cool boxes to the laboratory (<24 h). Samples were taken within a few hours on the same day in all plots. Once returned to the lab, we freeze-dried the samples and determined chlorophyll *a* content by acetone extraction (90%, dark, -20°C, 48 h) and methods described by Jeffrey and Humphrey (1975). The 2012 chlorophyll *a* concentration was measured with a spectrophotometer, whereas the 2015e16 samples were measured with a fluorometer.

For the organic matter samples, we took one sediment core (diameter: 2.6 cm, depth: 5 cm) at each transect point and in each experimental plot. The samples were placed into labeled plastic bags and stored in cool boxes on ice until they were transported to the laboratory. Upon return to the laboratory, the samples were frozen at -20°C until further processing. Organic matter content was measured by Loss on Ignition (LOI; 4 h, 550 °C) of oven dried (48 h, 60 °C) samples.

The dissolution plasters were collected after exposure to two tidal cycles and allowed to dry in air for a week. The relative plaster loss was then calculated by subtracting the dry weight of the plaster after exposure to the tides from the dry weight before exposure. Submersion time in the different plots only varied marginally, therefore no standardization was deemed necessary.

Photosynthetic yield was measured as the maximum quantum yield of photosystem II with a Pulse Amplified Modulation fluorometer (Mini-PAM, Walz) as a proxy for benthic microalgae productivity. To avoid differences caused by different

light conditions and changes in weather and timing of the tide, we collected triplicate samples of sediment at each transect point and transported them back to the lab (some were lost in 2012, reducing replication to duplicates at some points, $N = 30$). The samples were placed in petri dishes which were randomly distributed on the same shelf in a climate room and allowed to acclimatize for half a day (light level: $11 \mu\text{mol.m}^{-2}.\text{s}^{-1}$; temperature: 16°C). The photosynthetic yield was then measured using the PAM after 30 min of dark adaptation. The PAM sensor was fixed 10 mm above the sediment.

Statistical analysis

All statistical analyses were performed and graphs were created in R v 3.3.1 (R Core Team, 2017). If necessary, the data were logtransformed to meet the assumptions of homogeneity of variances and normality of data distribution.

Transects

Since the methods for the transect chlorophyll *a* data varied slightly between 2012 and 2015e16, we analyzed them separately. 2012 transect chlorophyll *a* concentration did not meet the assumption of homogeneity of variances, even after data transformation. Therefore, we analyzed differences in chlorophyll *a* concentration between the habitat types (mussel bed, no mussel bed) using a Kruskal-Wallis rank sum tests. We then performed two separate Spearman rank correlations for each habitat type, testing if chlorophyll *a* concentration changed with distance to the mussel bed (300 m, 200 m, 150 m, 100 m, 0 m). We averaged all three transects per habitat for the analysis. For the 2015-16 transect data of chlorophyll *a* and organic matter content, we performed analyses of covariance (ANCOVA) considering the factor habitat type (mussel bed, no mussel bed) and the covariable distance to the mussel bed (Table C.1 and Table E.1). For the analyses, we used data from all the six different sampling times in 2015e16. We pooled the 2012 and 2016 PAMdata for the analysis of photosynthetic yield, because lost samples of 2012 led to poor replication. We performed an ANCOVA, considering year (2012, 2016) and habitat type (mussel bed, no mussel bed) as the two factors and

distance to the mussel bed as the covariable (Table C.1). We used the triplicates per sampling point.

Field experiment

For the experiment, we analyzed the effect of the two different controls and mussel addition treatments on chlorophyll *a* concentration, organic matter content and hydrodynamic stress (plaster loss) using a fully crossed three-factorial ANOVA with site, cage and mussel addition as fixed factors.

RESULTS

Transect results

The mussel bed increased benthic microalgae biomass and productivity across the intertidal flat; both in the 2012 and the 2015-16 samplings (Fig. 1a-d).

On average, the chlorophyll *a* concentration was significantly higher in the mussel bed habitat compared to the no mussel bed habitat in both sampling periods (Fig. 1a-b; 2012: $X^2 = 9.62$, $df = 1$, $p = 0.002$; 2015-16: $F_{1,52} = 25.55$, $p < 0.01$, Table C.1). In 2012, the chlorophyll *a* concentration significantly increased with increasing proximity to the mussel bed in the mussel bed transect (Fig. 1a; $n = 18$, Spearman $R = 0.82$; $t = 5.8$, $p < 0.001$). In the no mussel bed habitat in 2012, there was no similar increase of chlorophyll *a* with distance to the tidal level of the mussel bed (Fig. 1b; $n = 18$, Spearman $R = 0.24$; $t = 0.97$, $p = 0.35$). The increase in benthic microalgae biomass directly on the mussel bed was on average more than 25 times stronger in the mussel bed transect compared to points at the same tidal elevation in the habitat without a mussel bed (2012; Fig. 1a). In 2015-16, the chlorophyll *a* concentration significantly increased with increasing proximity to the mussel bed and corresponding tidal level in both transects (Fig. 1b; significant main effect of distance: $F_{1,52} = 21.12$, $p < 0.01$, Table

C.1). The interaction between habitat type and distance to the mussel bed was statistically not significant (2015-16: $F_{1,52} = 1.76$, $p = 0.19$, Table C.1), but the increase was nearly four times stronger in the mussel bed transect compared to points at the same tidal elevation in the habitat without a mussel bed (Fig. 1b).

The effect of the mussel bed on photosynthetic yield depended on the year (Fig. 1c-d), as we detected a significant interaction between year, habitat type and distance to mussel bed ($F_{1,50} = 5.10$, $p = 0.03$, Table C.2). In 2012, the photosynthetic yield decreased gradually in seaward direction across the transect without a mussel bed while it increased strongly with proximity to the mussel bed in the mussel bed habitat (Fig. 1c). In contrast, in 2016 the photosynthetic yield increased gradually in seaward direction across the no mussel bed transect (Fig. 1d). The yield in the mussel bed transect in 2016 was in general higher than in the no mussel bed transect, but had an abrupt minimum 100 m coastward of the mussel bed, where the yield was even lower than in the no mussel bed habitat at a comparable tidal elevation (Fig. 1d).

Fig. 1. a) Chlorophyll a concentration 2012, b) chlorophyll a concentration 2015-16, c) photosynthetic yield 2012, and d) photosynthetic yield 2016 in the transects. Negative distances to the mussel bed indicate positions coastward of the mussel bed, whereas positive values indicate a position seaward of the mussel bed. In the no mussel bed habitat, the distances represent similar tidal elevations as in the mussel bed habitat. The gray circles represent averages of transects in the mussel bed habitat and the black circles represent averages of transects in the no mussel bed habitat. Error bars denote standard errors of the mean.

This area was characterized by the presence of macrofaunal structures (tube worms) that covered the entire area and possibly disturbed the biofilm. Overall, photosynthetic yield was higher in 2016 compared to 2012 (Fig. 1c-d; Year: $F_{1,50} = 36.41$, $p < 0.01$, Table C.2) and there was a marginal trend towards an average higher yield in the mussel bed habitat in both years (Habitat type: $F_{1,50} = 3.30$, $p = 0.08$, Table C.2). The highest yield overall was recorded in the mussel bed habitat for both years: in 2012 the highest yield was measured directly on the mussel bed (Fig. 1c); whereas in 2016 it was measured 100 m seaward of the mussel bed (Fig. 1d). The positive effect of the mussel bed on both biomass and productivity of the benthic microalgae resulted in a strong correlation

between chlorophyll *a* concentration and photosynthetic yield across the transects in June 2012 and June 2016 (including all matching chlorophyll *a* and yield measurements sampled from the same transect points in June 2012 and June 2016: Spearman rank correlation $r = 0.76$, $N = 30$, $p < 0.05$; Fig. 2).

Fig. 2. Correlation between photosynthetic yield and chlorophyll *a* concentration for both years (2012 and 2016). The gray circles represent data points in the mussel bed habitat, whereas the black points represent data points in the habitat without a mussel bed. We show data for all transect points that had corresponding chlorophyll *a* and yield measurements ($N = 30$).

Results field experiment

The mussel additions doubled chlorophyll *a* concentration in the sediment (Fig. 3a; Table 1). There was an indication of a stronger effect in the caged treatments, but there was no significant effect of caging, and no interaction effect between caging and mussel addition (Fig. 3a; Table 1). The mussel additions had no significant effects on organic matter content or plaster loss (Fig. 3b-c; Table 1). There was a marginal trend for the cage treatment to decrease plaster loss (Fig. 3c; Table 1), indicating that the cages decreased hydrodynamic stress in a way that the mussels alone did not.

The chlorophyll *a* concentration of the sediment was higher at site 2 coastward of the mussel bed, compared to site 1, without a mussel bed (Fig. D.1a; Tukey HSD post hoc test: site 2 > site 1, $p < 0.05$); but there was no interaction between the experimental treatments and site, demonstrating that mussels increased chlorophyll *a* concentration across habitat types (Table 1). The organic matter content was highest at site 2 (behind the smaller mussel bed) and lowest at site 1, without a mussel bed (Fig. D.1b; Tukey HSD post hoc test: site 2 > site 3 > site 1, $p < 0.05$). There was a nonsignificant trend for decreased hydrodynamic stress at site 3 compared to the other sites (Fig. D.1c).

Fig. 3. a) Average chlorophyll a concentration, b) average organic matter content (OM), and c) average plaster loss in the different treatments (FC = fenced control, FM = fenced mussel addition, CC = semi-caged control, CM = semi-caged mussel addition) of the field experiment. Bars denote standard errors of the mean.

Table 1. Results of three-factorial ANOVA for field experiment testing effects of mussel additions (two levels - no mussel addition, mussel addition), cage (two levels - cage present, no cage present) and site (three levels); on chlorophyll *a* concentration (Chl *a*), organic matter content (OM) and plaster loss (PL) in the experimental plots.

Factor	df	Chl <i>a</i> (µg/mg)		OM (%)		PL (%)	
		F	p	F	p	F	p
Mussel addition (M)	1	12.59	<0.01	1.71	0.20	0.66	0.43
Cage (C)	1	0.52	0.48	0.22	0.65	4.53	0.05
Site (S)	2	5.45	0.01	16.00	<0.01	0.88	0.43
M x C	1	0.37	0.55	3.14	0.09	1.46	0.24
M x S	2	0.21	0.81	1.28	0.30	0.92	0.41
C x S	2	0.73	0.49	0.70	0.51	3.40	0.05
M x C x S	2	0.66	0.53	0.82	0.46	0.23	0.80
Residuals	2						

DISCUSSION

We demonstrate that mussel beds increase the biomass of primary producers. Previous research had suggested this facilitative effect of mussel beds (Donadi et al 2013b), but did not provide conclusive evidence. Our results are also the first to actually link a long-distance interaction of up to 200 m in the vicinity of mussel beds, to both increasing biomass of primary producers and higher levels of productivity. There was a clear inter-annual variation in both chlorophyll *a* and productivity, where the long-range effect of the mussel bed shifted in magnitude and direction between 2012 and 2015-16. However, in general, the areas on the tidal flat with the highest biomass of benthic microalgae also had high values of photosynthetic yield and these were always the sampling points in proximity of the mussel bed; indicating that the positive effect of the mussel bed prevailed through the annual fluctuations in environmental conditions. These results confirm and highlight the importance of reef-building bivalves for the functioning of coastal ecosystems. Specifically, in soft-sediment habitats reef-building bivalves act as ecosystem engineers and thereby are essential for associated communities (van de Koppel et al 2015). By creating a three-dimensional habitat structure, bivalve reefs are important structuring components of many coastal ecosystems. In soft bottom intertidal areas, bivalve reefs are often the only hard surfaces that can be used by other sessile organisms for attachment, favoring habitat forming algae and rich communities of epifauna (Albrecht and Reise 1994). Bivalve reefs are also important nursery grounds for many economically important organisms such as fish (Kristensen et al 2015). In addition to locally modifying the habitat with their physical presence, they also have long distance effects by creating sediment conditions around them that are beneficial for many benthic organisms including infauna species (van der Zee et al 2012; Donadi et al 2015). This shows that bivalves are autogenic and allogenic ecosystem engineers at the same time (Jones et al 1994). Our study demonstrates that mussel beds not only facilitate animal biodiversity on many different trophic levels as shown in previous studies (Albrecht and Reise 1994; van der Zee et al 2012; Donadi et

al 2015; Kristensen et al 2015), but they also provide excellent growing conditions for benthic primary producers across the intertidal. Together, this demonstrates that mussel beds are hot spots that fuel primary production in intertidal areas and support a productive and diverse food web far beyond its physical borders.

The abundance of benthic microalgae in intertidal sediments strongly depends on sediment erosion. Increased hydrodynamic stress leads to higher sediment erosion, which in turn re-suspends the microalgae (de Jonge and van Beusekom 1995). Larger biofilms of benthic microalgae are therefore often found in areas that are protected from hydrodynamic stress. Previous research shows that mussel beds reduce hydrodynamic stress (Donadi et al 2013b) and therefore productivity of benthic primary producers is expected to increase in the vicinity of these structures. Our transect results confirm an increase in primary producer biomass closer to the mussel bed, but in our experiment we found that protection from hydrological forces alone does not increase the standing stock of primary producers. Although shelter by the cages decreased the hydrodynamic stress more than the mussels, it was only when mussels were physically present in the plots we could demonstrate an increase in primary producer biomass. Generally, in addition to reducing hydrodynamic stress, mussels excrete feces and pseudofeces that are rich in organic matter and nutrients which may promote growth of the biofilm (van Broekhoven et al 2015). The physical changes by the mussels may also hinder infauna that feed on benthic microalgae and/or affect sediment stability via bioturbation. For example, the amphipod *Corophium volutator* and the polychaete *Arenicola marina*, are common infauna found in the Wadden Sea that negatively affect benthic microalgae via grazing and bioturbation, respectively (Gerdol and Hughes 1994; Chennu et al 2015). Thus, the exclusion of infauna grazers and bioturbators may also have promoted the biofilm (Gerdol and Hughes 1994; Brustolin et al 2016). However, the reefs also support higher abundances of epifauna (Norling and Kautsky 2007), which includes dominant grazers such as *Littorina littorea* (common periwinkle) that consume the biofilm. Consequently, in our experiment, on a local scale, the increase in microphytobenthos

biomass by the physical presence of the mussels may have been caused through a combination of: (1) reduced hydrodynamic stress, (2) increased nutrient availability, and (3) changes to the associated invertebrate community.

Scale is important for the magnitude of engineering effects. Our experiment was designed to analyze effects at small scales and therefore did not simulate the hierarchical spatial structure of mussel beds or manipulate long-range effects (Liu et al 2012; van de Koppel et al 2015). The hierarchical structure of the mussel patches possibly play a major role on the overall engineering effect of mussel beds on the tidal flats systems. Indeed, comparing the experimental results of mussel addition to the field measurements across the mussel bed indicates that the facilitative effect on primary producers was dependent on the extent of the mussel aggregation. Our small addition plots doubled the biomass of benthic microalgae, but this was still less than half of the biomass on the natural mussel bed. The importance of the size of mussel patches was previously observed for associated macrofauna (Norling and Kautsky 2007). Even though the presence of single mussels was shown to increase biomass and species richness of associated macrofauna, the increase was much higher in larger patches of *Mytilus edulis* (Norling and Kautsky 2007). The temporal scale is probably also very important; the mussel bed from the transect data had been present in the same location for multiple years before we took the measurements. This means that there was much more time for organic matter to be accumulated in the sediment and we saw a large increase of organic matter content on the mussel bed (Fig. E.1). In contrast, we did not see any significant effect of the mussel additions on organic matter content in the experimental plots, where the mussels only had about one month time to produce and accumulate organic matter. Worldwide, we have observed a decrease in native bivalve reefs due to overfishing, habitat degradation, or invasion of non-native species (Jackson et al 2001; Edgar and Samson 2004; Lotze et al 2006; Eriksson et al 2010), which can have detrimental effects on coastal ecosystems. Protection and restoration efforts should

not only focus on the presence of the species but also needs to consider the scale of reefs.

Our results demonstrate the importance of bivalve reefs for primary production on intertidal flats. On a global scale, we see a decline in ecosystem engineering species in coastal areas (Jackson et al 2001; Lotze et al 2006). Bivalves are therefore already of an importance to conservation and restoration efforts in many areas of the world (e.g. Schulte et al 2009; McLeod et al 2012; de Paoli et al 2015). Our research shows that establishing and protecting reefs in soft-bottom habitats is a key conservation priority and an essential strategy to restore and manage coastal production.

ACKNOWLEDGEMENTS

We thank Vereniging Natuurmonumenten and the Province of Friesland for granting us permission for field work on the tidal flats. Special thanks to Luna van der Loos and Just van der Endt for their help setting up the field experiment; Stella Boele-Bos for help in the laboratory; the 2012 Marine Research students Reinier Nauta, Karsten Roelfsema, Melanie Timmerman, Bart Zwart and the 2016 Marine Biology Research students Alex Hanewacker, Sjoerd den Hertog, Titus Hielkema, Jorick Hiemstra for help collecting the transect data. This work was supported by a grant from the ZKO program of the Netherlands Organization of Scientific Research (NWO) to BKE (grant no. 839.08.310).

Appendix A3.1 Detailed information on study site (transects and experiment)

Figure A.3.1.1 a) Overview of the study areas for the transect measurements as well as for the experiment; (a) close-up of transect set-up; (b) close-up of experimental set-up, (c) with the four treatments fenced control (FC), semi-caged control (CC), fenced mussel addition (FM), and semi-caged mussel addition (CM).

Appendix A3.1 (continued)**Table A.3.1.1** *Coordinates and attributes of transect sampling points.*

Transect	Plot	Plot ID	Habitat	Latitude (°N)	Longitude (°E)	Distance MB (m)
2	A	2A	Mussel bed	53.47090	6.22400	-350
2	B	2B	Mussel bed	53.47045	6.22408	-300
2	D	2D	Mussel bed	53.46955	6.22418	-200
2	F	2F	Mussel bed	53.46865	6.22438	-100
2	H	2H	Mussel bed	53.46776	6.22462	0
2	J	2J	Mussel bed	53.46686	6.22469	100
2	K	2K	Mussel bed	53.46494	6.22492	150
5	A	5A	No Mussel bed	53.47101	6.23025	-350
5	B	5B	No Mussel bed	53.47055	6.23029	-300
5	D	5D	No Mussel bed	53.46967	6.23041	-200
5	F	5F	No Mussel bed	53.46876	6.23050	-100
5	H	5H	No Mussel bed	53.46788	6.23058	0
5	K	5K	No Mussel bed	53.46521	6.23065	150

Appendix A3.1 (continued)

Figure A.3.1.2 a) Example of experimental set-up (depicted is part of Site 1); b) fenced control treatment (FC); c) fenced mussel addition treatment (FM); d) semi-caged control; e) semi-caged mussel addition treatment.

Table A.3.1.2 Coordinates for set-up sites of the field experiment.

Site	Latitude (°N)	Longitude (°E)
1	53.28590	6.13474
2	53.28720	6.13293
3	53.28775	6.11262

Appendix A3.2 Cage effect analysis for the experiment

Figure A.3.2 Average chlorophyll a concentration (a) and organic matter content (OM; b) in the different cage treatments of the experiment (Control, FC = fenced control, CC = semi-caged control) of the field experiment. Bars denote standard errors of the mean.

Appendix A3.2 (continued)

Table A.3.2.1 Results of two-factorial ANOVA for cage effect in field experiment. We tested the influence of the factors Treatment (Tr; three levels – Control, Fence, Cage) and Site (S; three levels) on the response variables chlorophyll a and organic matter content.

Response Variable	Factor	df	SS	F	p
Chlorophyll a	Tr	2	8.72	0.36	0.71
	S	2	200.16	8.14	<0.01
	TrxS	4	16.59	0.34	0.85
	Residuals	15	184.35		
Organic Matter	Tr	2	0.37	0.62	0.55
	S	2	1.65	2.73	0.10
	TrxS	4	0.79	0.65	0.64
	Residuals	15	4.53		

Table A.3.2.2 Post-hoc results (Tukey HSD) for cage effect in the field experiment. We compared chlorophyll a concentration and organic matter content in the different sites and in the different cage treatments additions.

Response Variable	Pair	p
Chlorophyll a	Site1:Site2	<0.01
	Site1:Site3	0.44
	Site2:Site3	0.05
	Control-Fence	0.73
	Control-Cage	1.00
	Fence-Cage	0.79
Organic Matter	Site1:Site2	0.10
	Site1:Site3	0.22
	Site2:Site3	0.91
	Control-Fence	0.57
	Control-Cage	0.99
	Fence-Cage	0.70

Appendix A.3.3 ANCOVA tables for transect data 2012 and 2015-16.

Table A.3.3.1 Results of ANCOVA for transect chlorophyll a data 2015-16. We considered the factor habitat type (HT; two levels – no mussel bed and mussel bed) and the covariate distance to mussel bed (D; continuous) and the interaction HTxD. We used the log-transformed data from all six sampling.

Response Variable	Factor	df	SS	F	p
<i>Chlorophyll a 2015-16</i>	HT	1	2.47	25.55	<0.01
	D	1	2.04	21.12	<0.01
	HTxD	1	0.17	1.76	0.19
	Residuals	52	5.02		

Table A.3.3.2 Results of ANCOVA for transect PAM data. We considered the factors habitat type (HT; two levels – no mussel bed and mussel bed) and year (Y, two levels – 2012 and 2016) and the covariate distance to mussel bed (D; continuous) and all interactions. We used triplicate samples per transect point.

Response Variable	Factor	df	SS	F	p
<i>Photosynthetic Yield</i>	HT	1	0.02	3.30	0.08
	D	1	<0.01	0.02	0.89
	Y	1	0.20	36.41	<0.01
	HTxD	1	0.03	5.10	0.03
	HTxY	1	0.02	3.30	0.08
	DxY	1	<0.01	0.02	0.89
	HTxDxY	1	0.03	5.10	0.03
	Residuals	50	0.27		

Appendix 3.4 Average chlorophyll a, organic matter, and plaster loss per site.

Figure A.3.4.1 a) Average chlorophyll a concentration (Chl a), b) average organic matter content (OM), and c) average plaster loss in the different sites of the field experiment. Bars denote standard errors of the mean.

Appendix A.3.5 Organic Matter Content in the Transects

Figure A.3.5.1 Organic matter content (OM) in the transects at different sampling points. Displayed are means of the six sampling times in 2015-2016 with standard errors

Appendix A.3.5 (continued)

Table A.3.5 Results of ANCOVA for organic matter in the 2015-16 transects. We considered factor habitat type (HT; two levels – no mussel bed and mussel bed) and the covariate distance to mussel bed (D; continuous) and the interaction HTxD. We used log-transformed data from all six sampling times.

Response Variable	Factor	df	SS	F	p
<i>Organic Matter</i>	HT	1	0.42	21.45	<0.01
	D	1	0.60	30.41	<0.01
	HTxD	1	<0.01	0.11	0.74
	Residuals	52	1.03

#Biofilm at Intertidal flat of Sciermonnikoog (©R. Andriana)

**Between stimulus and response,
There is a space where we choose our response.**
-Stephen Covey-

CHAPTER 4

Intertidal mussel reefs change the composition and size distribution of diatoms in biofilm

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ABSTRACT

Migrating diatoms are microscopic ecosystem engineering organisms that have functional consequences on the seascape scale by significantly contributing to the microphytobenthos bioilm. The microphytobenthos bioilm is a thin photosynthesising layer that covers the sediment on intertidal lats. It fuels the food web, increases sediment stability, and enhances the deposition of particles, providing ecosystem services to coastal communities. Here we tested the effect of another ecosystem engineering habitat, intertidal blue mussel reefs, on the composition and properties of migrating diatom communities. Small-scale reefs constructed in the intertidal mimicked and reinforced the natural pattern in diatom community composition and function that we documented in the field. The field experiment adding small reefs to the intertidal ran from 30 April to 10 June 2015 and the field samples were collected around a natural blue mussel bed on the same tidal lat on 7 October 2015 (N 53.489°, E 6.230°). Both the constructed small-scale reefs and the natural reef changed the community composition of diatoms in the biofilm by promoting higher numbers of smaller-sized cells and species. Small diatoms have higher growth and gross photosynthesis rates, indicating that this explains the higher production and chlorophyll *a* concentration of the biofilm measured on natural intertidal shellfish reefs. Our results showed that shellfish reefs have a large impact on biofilm functioning. However, biofilms are also fuel for the shellfish, indicating that the two very diferent ecosystem engineers may facilitate coexistence on tidal flats through a positive feedback loop.

Marine Biology (2021). **168**, p.24.

<https://doi.org/10.1007/s00227-020-03819-2>

INTRODUCTION

Marine soft sediment diatoms are microscopic ecosystem engineers that fundamentally alter the function of intertidal flats. They are characterised by two main groups of motile pennate raphid diatoms: epipelon, which is larger (length of $> 10 \mu\text{m}$) and moves freely in the muddy sediment, and epipsammon with smaller size (length $< 10 \mu\text{m}$) that can be found closely associated with sandy grains (Barnet et al 2015; Ribeiro et al 2013). The intertidal diatom community is easily recognised through their significant contribution to a thin-brown photosynthesising layer, the biofilm, that forms on top of the sediment during low tide (Aleem 1950; Kromkamp and Foster 2006). The biofilm consists of a complex matrix of extracellular polymeric substances (EPS) that surrounds the biofilm-associated organisms. The EPS is produced in large quantities by motile diatoms and has an important ecosystem function by binding particles together; stabilising shallow sediment; and influencing sediment transport on the seascape scale (Grant et al 1986; Vos et al 1988; Paterson 1989; Hoagland et al 1993; Malarkey et al 2015).

Primary productivity of the biofilm on the intertidal flat is a result of hydrodynamic disturbance (regulating sediment erosion), sediment characteristics (organic matter content, grain size), the elevation of the tidal flat (influencing the photoperiod), species composition (influencing pigment composition and growth rates), and vertical migration of the benthic diatoms themselves (Heip et al 1995; De Jong and Jonge 1995; Paterson 1986). The diatom biofilm contributes to a significant part of the total primary production on intertidal flats and fuels the benthic food web by supporting higher trophic levels, including blue mussels, oysters, birds, and fish (Admiraal 1984; Underwood and Kromkamp 1999; Christianen et al. 2017).

Intertidal blue mussel reefs (*Mytilus edulis* L.) are themselves important ecosystem engineers that mitigate hydrodynamic disturbance, change sediment properties, and provide habitat for a variety of associated organisms across trophic

levels (Widdows et al 1998, Widdows and Brinsley 2002; Gutiérrez et al 2003; van der Zee et al 2012). The engineering effects of blue mussels manifest both within the reefs and on a larger scale; blue mussel reefs modify the surrounding up to several hundred meters across the tidal flat (Donadi et al 2013a; van de Koppel et al 2015). The mussels remove suspended particulate matter from the water column and deposit this as nutrient-rich faeces and pseudofaeces (Biodeposit: Verwey 1952; Kautsky 1987; Asmus and Asmus 1991). Biodeposit from bivalves display elevated nutrient and chlorophyll *a* concentrations, indicating that the enrichment of the sediment contributes to an increased diatom production (Giles and Pilditch 2006; Donadi et al 2013b). In addition, shelter provided by intertidal blue mussel reefs can initiate the development of diatom films in areas where the energy is otherwise too high (Donadi et al 2013b; Engel et al 2017). When a biofilm starts to develop in an area, EPS accumulation on the surface of the sediment will bind smaller sediment particles and decrease the sediment erosion threshold, increasing diatom production and organic matter content (van de Koppel et al 2008). Diatoms grow better in sediment with high organic matter content because of the correlation with low sediment erosion and high nutrient availability (Stal and Brouwer 2003). Van de Koppel et al (2008) showed that diatoms exude EPS that stabilises the sediment, which further promotes diatom growth and even more EPS production, leading to a positive feedback that promotes a stable and productive biofilm. Similarly, bivalves are favoured by low sediment erosion because this promotes the settlement and establishment of their juveniles (Donadi et al 2014; van de Koppel et al 2015). Because of resuspension, the microphytobenthos is also an important food-source for blue mussel reefs on tidal flats. Thus, while blue mussel reefs are fuelled by the biofilm, they also facilitate the biofilm, indicating that positive feedbacks between these very dissimilar ecosystem engineers may reinforce each other (Eriksson et al. 2010).

In this study, we tested the effect of intertidal blue mussel reefs on the biodiversity and composition of the migrating diatom community, to increase the understanding of the interaction between bioengineering of the mussels and the function

of the biofilm. In earlier studies, we have observed that the production and chlorophyll-*a* content of the biofilm increase on mussel reefs compared to bare areas on an intertidal flat (Engel et al 2017). The size of diatoms (measured as biovolume) is inversely related to their growth rates and the chlorophyll-*a* specific absorption coefficient, which is an important property determining gross photosynthesis rates (Geider et al 1986; Morin et al 2008). Small diatoms also have a relatively large surface to volume ratio, which allows them to absorb nutrients rapidly and compete successfully in nutrient rich environments (Geider et al 1986). We therefore hypothesised that the increase in biofilm production observed on mussel reefs (Engel et al 2017) relates to a shift in diatom community composition towards smaller species and a higher total biovolume caused by an increased number of cells. Changes in the growth rate, composition of species, and size distribution of the diatom community influence EPS production (Smith and Underwood 1998; Underwood and Smith 1998; Brouwer et al 2005) and therefore should have consequences for the sediment stabilising function of the biofilm. Intertidal blue mussel reefs often form large aggregations that span 100s of meters, and consist of a thick and elevated mussel matrix interspersed by smaller patches of bare substrate and water-filled pools. Since it is difficult to construct or manipulate such a complex and large structures as natural blue mussel beds in a realistic way, we instead created small plot-sized (1 m²) artificial mussel reefs to causally link the changes in the migrating diatom community to the presence of mussels. Since blue mussel reefs enrich the sediment and decrease sediment erosion, we hypothesised that the small-scale additions of mussels would promote local production and development of the biofilm: generating a biofilm with higher diatom biovolume, with higher diatom abundances, and with a higher fraction of smaller diatom species, compared to plots where we did not add mussels (e.g. Asmus and Asmus 1991; Geider et al 1986; Giles and Pilditch 2006; Donadi et al 2013a). However, the small-scale artificial reefs do not mimic the large-scale hydrodynamic effects and complexity of natural blue-mussels reefs (e.g. Donadi et al. 2013a). To make a realistic inference of the results, we therefore compared the

results from the small-scale experiment with the natural species composition and size distribution of the migrating diatom community around a large intertidal shellfish reef.

MATERIAL AND METHODS

Field experiment

We conducted the field experiment on an intertidal flat in the Netherlands, south of the island of Schiermonnikoog (N 53.489°, E 6.230°, Fig. S1). The intertidal flats in this area are characterised by vast stretches of bare sediment that are occasionally broken up by blue mussel reefs that can cover several hectares. In the past decade, the Pacific oyster (*Crassostera gigas* [Thunberg]) has increased strongly on the blue mussel reefs (Andriana et al. 2019). Today, the older parts of the reefs consist of a mixed matrix of oysters and blue mussels, while the younger parts are still completely dominated by blue mussels. Previous studies have shown that benthic habitat characteristics (e.g. sediment type) vary depending on proximity to shellfish reefs (Donadi et al. 2013a, b). We therefore replicated the experimental set-up in two sites with visibly different sediment character, one on the coastal side of the mussel reef (along field study transect MB, see field study below; site “wake”) and one at the same tidal elevation in the sandy area adjacent to the reef (along field study transect S, see field study below; site “sand”) (Fig. S1). The “wake” site was more sheltered, and the sediment type (mud/silt, high organic matter content; see results) had a characteristic of cohesive sediment (plaster erosion loss: $12.8 \pm 1.3\%$ per tide, $n = 4$; relative shear strength of the sediment: 7.5 ± 2.7 , $n = 7$; mean $\pm$ SD). The “sand” site was more exposed and had a sediment type characteristic of sandy less cohesive sediment (plaster erosion loss: $14.9 \pm 1.2\%$ per tide, $n = 4$; relative shear strength of the sediment: 2.5 ± 2.2 , $n = 7$; mean $\pm$ SD). Erosion was measured as weight loss of model plaster (Knauf Modelgips, Knauf B.V., Utrecht, The Netherlands) moulded in plastic cylinders (6.3 cm long; 2.4 cm diameter) with a pin through them. The pin was inserted in the sediment so that the plaster cylinders were

fully exposed to the incoming water. The cylinders were left on the tidal flat for two tidal cycles and then dried in the laboratory until constant weight. We measured the undrained shear strength of the sediment using a handheld shear vane. A shear vane consists of a rod with vanes mounted to one end and a torque gauge in the other end. The vane is inserted in the sediment and slowly rotated until the cohesiveness of the sediment fails, so that the vane rotates in the sediment (Grabowski 2014). However, vanes are normally adapted for soil with high shear strength. We therefore used longer home-made and non-calibrated vanes in all our samplings, which are more suitable for softer intertidal sediment (pers comm Tjeerd Bouma, NIOZ). Thus, we did not transform the shear strength to an absolute unit. The measurements are therefore limited to show differences in relative cohesion between sites. The measurements were collected across the whole tidal flat on two different occasions in September and October by different persons. We, therefore, min-max normalised the data to be between 0 and 10 to make it comparable.

At each site, we constructed 15 experimental plots with an area of 0.25 m² each (sides 0.5 by 0.5 m), placed in three parallel rows with three meters between each of the plots (to all sides). To six randomly chosen plots at each site, we added live blue mussels (*M. edulis*) (treatment “reef”), while six other plots were left unaltered (treatment “bare flat”). Placing experiments on an intertidal flat may lead to critical artefacts because of changes to water flow caused by equipment rather than treatments, but also because of changed predation rates since the experiment may hinder or attract natural predators. Isolated clumps of blue mussels are highly prone both to crab and bird predation and may quickly be completely removed when placed on the tidal flat. We therefore protected the mussels by constructing two types of experimental controls with different effects on predation and exposure. Half of the plots in both the bare flat and reef treatments had a fence mounted around them consisting of a plastic net (“plastic fence”). The plastic net protects the mussels from both crab and bird predation but may have strong effects on flow. The other half of the plots had a fence consisting of a rope placed

around the perimeter that only hinder birds from eating the mussels but have minimal effects on flow (“rope fence”). The ropes were fastened in a 66-cm long plastic pole marking each corner of the plot, inserted 30 cm into the sediment. In the bare flat treatments, the same types of fences (cage and rope) were used, to have a comparable control to the mussel reef treatment. In addition, three plots at each site were left completely unmanipulated (no addition of mussels and no addition of a fence), to separately test for “fence” effects. We did this by comparing the effects of fence on the diatom community in the plots without mussel additions: thus comparing the “no mussel additions and no fence” treatment (unfenced control) with the “bare flat and plastic fence” and “bare flat and rope fence” treatments, respectively (see Appendix 4.2). The experiment was set out on the 30 April 2015. We sampled the sediment in the plots to analyse diatom abundance and species composition, chlorophyll-*a* concentrations, and organic matter content, on the 10 June 2015, approximately 1 month after the mussel addition and at the height of biofilm development. Diatom abundance as estimated by chlorophyll-*a* concentrations in the sediment normally peaks in the month of June in the Wadden Sea (Staats et al 2001; Van der Wal et al 2010). For more details on the experimental set-up, see Engel et al 2017 (Chapter 3).

Field study

We performed the field study by setting up four parallel transects, spanning from the coast seaward, on the same intertidal flat as the field experiment (Fig. A.4.1). The transects were 200 m apart from each other and 400 m long. One transect crossed an intertidal blue-mussel reef (transect MB), two transects crossed the flat 50 m on each side of the shellfish reef (transects ME and MW), and one transect crossed a bare part of the flat 250 m from the shellfish reef (transect S). Previous analyses demonstrate a small spatial variability among sampling points based on their physical locations in relation to the mussel bed (Engel et al. 2017). We therefore divided all sampling points into either “reef front”: sites on the seaside close to the reef (50 m away; $n = 3$); “reef wake”: sites in the sheltered wake close to or on the reef (0–75 m; $n = 4$); or “bare sand”:

sites more than 100 m from the reef with visibly much sandier sediment than the sites closer to the reef ($n = 10$). We sampled sediment from each of the 17 transect points on the 7 October 2015; to analyse diatom abundance and species composition, chlorophyll-*a* concentration, and organic matter content (for “Material and methods” see below).

Sediment sampling and sample processing

At each transect point and in each experimental plot, we took sediment cores (diameter: 26 mm) for measuring chlorophyll-*a* concentration and organic matter content in the sediment, as well as to determine benthic diatom composition and biomass. For chlorophyll-*a* and species composition, we pooled the sediment from three cores of 0.2 cm (chlorophyll *a*) and 2 cm (diatom composition) depth, respectively. The cores were sampled 1 dm from each other in a triangular pattern, and the sediment was collected in a piece of aluminium foil to avoid exposure to light. Organic matter was sampled with a 5-cm-deep core (once in each plot/transect point). All samples were placed in sealed plastic bags and transported back to the laboratory on ice in a cool box. Further sample processing took place in the laboratory. Chlorophyll-*a* concentration was determined by a method derived from Jeffrey and Humphrey (1975): After freeze-drying the sediment, we extracted the chlorophyll with 90% acetone (dark, -20°C , 48 h) and measured concentration with a fluorometer (Trilogy, Turner Design). We determined organic matter content of the sediment by Loss on Ignition (burning oven dried samples in a muffle kiln at 550°C for 4 h). For diatom species identification and biovolume calculation, the live motile benthic microalgae were extracted from the sediment by spreading out the sediment in petri dishes and placing two layers of lens cleaning tissue on top of the sediment. After 5 h of exposure to light, we removed the top layer of the tissues, which now contained the diatoms that migrated to the surface. We rinsed the diatoms off the tissues with sterile North Sea water and collected the communities in brown glass bottles. We fixed the samples with Lugol's iodine solution and stored them in the dark until further processing. To determine species composition and biovolume of the diatoms, we used the Utermöhl technique (Utermöhl 1958) and

counted diatom cells under an inverted microscope (400 × magnification). We identified diatom groups to the highest resolution possible, which for some diatoms was the genus level. We calculated species-specific biovolumes based on methods described by Hillebrand et al (1999), which uses simple geometric shapes to approximate diatom cell volume by measuring multiple dimensions of several cells per species.

Data analyses

We analysed experimental effect on species composition based on both biovolume and cell numbers, using permutational multivariate analysis of variance (PERMANOVA) (analysing distance matrices using the `adonis2` function in the `vegan` package, version 2.5–6; R Core Team 2020; Oksanen et al. 2019). We analysed experimental effect on total biovolume, total cell number, average cell size, and species richness of the diatom community, as well as organic matter content of the top 5 cm of the sediment using analysis of variance (ANOVA). All models contained the fixed explanatory factors mussel addition (“bare flat” or “mussel reef”), type of fence (plastic net or rope), and site (reef “wake” or “sand”). However, we lost two of the fenced replicates at the sandy site creating an unbalanced design. We therefore compared AICc values for the univariate models including different levels of interactions (full factorial model: $\text{site} \times \text{fence} \times \text{mussel addition}$; two-way factorial model: $\text{site} \times \text{fence} + \text{fence} \times \text{mussel addition} + \text{site} \times \text{mussel addition}$; main effects model: $\text{site} + \text{fence} + \text{mussel addition}$) using ANOVA for unbalanced designs (Type III ANOVA using the `car` package in R, version 3.0–10). In the ANOVA analyses of total biovolume, total number of diatom cells, size distribution, and organic matter content, none of the interaction effects were statistically significant and the reduced model without any interactions was consistently the best model according to AICc (lowest AICc values; Table. A.4.3). For all univariate models (ANOVAs), except for species richness, we therefore present results from the most reduced model (main effects model). For species richness, we present results from the two-way factorial model, which was the best model according to AICc (Table A.4.3). If needed to fit assumptions of the models, data were sqrt- or

logtransformed. We did not analyse experimental effects on chlorophyll-*a* concentrations in the sediment, since these were analysed and presented for a larger subset in Engel et al (2017).

For the field study, the species composition based on biovolume data was analysed with Nonmetric Multidimensional Scaling (NMDS) using Bray Curtis dissimilarities (monoMDS in the vegan package, version 2.5–6; R Core Team 2020; Oksanen et al. 2019). We tested if there was a difference in community composition between different habitats (“reef front”, “reef wake”, or “sand”) using analysis of similarities (ANOSIM; vegan package, version 2.5–6; Core Team 2020; Oksanen et al. 2019). The relation between different covariates (total biovolume, cell numbers, average cell size and species richness of the diatom community, chlorophyll-*a* concentration, and organic matter content of the sediment) and the species composition of the diatom community was evaluated using the envfit function, which correlates the covariates onto the ordination axes. To get a visualisation of changes in species composition, we also plotted the weighted averages for each species along the ordination axes, based on the biovolume at each site (species scores). We also tested differences in the covariates between different habitats (“reef front”, “reef wake”, or “sand”) using one-way analysis of variance (ANOVA). If needed to fit assumptions of the models, data were sqrt- or log-transformed.

RESULTS

Field experiment

The experimental blue mussel reefs changed the species composition and size distribution of motile diatoms in the biofilm (Fig. 1). The community included 22 species groups of motile diatoms (Table A.4.4). There was a difference in diatom species composition between the plots with artificial mussel reefs compared to the plots without reefs (“bare flat”), and between the reef wake and sand plain habitats, both for the multivariate analyses based on biovolume and cell numbers (PERMANOVA; biovolume—mussel addition: $F_{1,14} = 2.8$, $p = 0.036$; site, $F_{1,14} = 7.1$, $p < 0.001$; cell number—mussel addition: $F_{1,14} = 2.8$, $p = 0.048$; site: $F_{1,14} = 8.0 < 0.001$; Fig. 1a, b). The change in community composition was mainly caused by the mussels promoting a higher number of diatoms with small cell sizes (biovolume $< 51 \mu\text{m}^3$; [CYL, NAV I&II, NITZ I&II]; Table A.4.4; Fig. 1a, b). The PERMANOVAs showed no significant interaction effects ($F_{1,14} < 1.5$, $p > 0.17$) and no significant effects of different types of fencing around the experimental plots (biovolume—fence: $F_{1,14} = 1.6$, $p = 0.18$; cell number—fence: $F_{1,14} = 1.9$, $p = 0.102$). In addition, there were no significant effects of the ropes or the plastic fence compared to the unmanipulated control (“fence” effects; Appendix A.4.2). The experimental reefs increased the total number of cells and decreased the average cell size of the diatoms (ANOVA, main effect of mussel additions—total cell number: $F_{1,18} = 4.6$, $p = 0.046$; average cell size: $F_{1,18} = 7.4$, $p = 0.014$), but had no significant effects on total biovolume, organic matter content of the sediment, or species richness (ANOVA, main effect of mussel additions—total biovolume: $F_{1,18} = 0.7$, $p = 0.417$; organic matter content: $F_{1,18} = 0.1$, $p = 0.768$; species richness: $F_{1,18} = 0.7$, $p = 0.417$; Table A.4.5, Fig. 1). The total biovolume, total cell numbers, organic matter content of the sediment, and species richness were higher in the wake of the mussel bed compared to the sand plain habitat (ANOVA, main effect of site—total biovolume: $F_{1,18} = 11.1$, $p = 0.004$; total cell number: $F_{1,18} = 17.1$, $p < 0.001$; organic matter content: $F_{1,18} = 36.7$, $p < 0.001$; species richness: $F_{1,18} = 30.7$,

$p < 0.001$; Fig. 1). Site had no effect on average cell size (ANOVA: $F_{1,18} = 0.8$, $p = 0.373$). The type of fence only had significant effects on species richness (Table A.4.5), where the plastic fence increased species richness in the more sheltered wake of the mussel bed (ANOVA, significant interaction effect between fence type and site: $F_{1,18} = 11.0$, $p = 0.005$). There were no significant “fence” effects on any of the response variables, comparing different fences to the unmanipulated control (Appendix 4.2).

Field study

We found a clear difference in the diatom community composition between the sand habitat, and the front and wake of the mussel reef (Fig. 2). According to the analysis of similarities (ANOSIM), the variation in species composition was statistically significantly different between different habitats ($R = 0.43$, $p = 0.004$; Fig. 2a). *Navicula* spp. dominated the biofilm, but the reef front was additionally characterised by the largest species: 56% of the total biovolume consisted of species with a biovolume $> 1300 \mu\text{m}^3$ (*Pleurosigma aestuarii*, *Entomoneis paludosa*, *Stauroneis* spp., and the largest species of *Navicula* [NAF and NAA; Table S4; Figs. 2b, 3a). The reef wake was characterised by high numbers of the smaller species: 57% of the counted individuals had a biovolume $< 51 \mu\text{m}^3$ (*Cylindrotheca closterium*, and the smallest species of *Navicula* [NAV I&II] and *Nitzschia* [NITZ I&II]; Figs. 2b, 3b). The sand communities were completely dominated by medium sized *Navicula* species: 92% of the biovolume (NAV III&IV, Fig. 3a). There was a significant correlation between total biovolume, cell size, chlorophyll-*a*, organic matter content of the sediment, and species richness, and the composition of the diatom community along the ordination axes (Fig. 2a) (NDMS, envfit function results: total biovolume: $r^2 = 0.57$, $p = 0.004$; cell size: $r^2 = 0.53$, $p = 0.006$; chlorophyll-*a*: $r^2 = 0.38$, $p = 0.046$; organic matter: $r^2 = 0.41$, $p = 0.031$; species richness: $r^2 = 0.67$, $p < 0.001$; Fig. 2a). Statistical analyses of differences between habitats supported some of the apparent relations in the NDMS plot: the average size of the diatom cells and total biovolume was highest in the mussel reef front; the species richness of the diatom film was higher in both reef affected habitats (“reef

front” and “reef wake”) compared to the sand flat; and there were no significant differences in total number of diatom cells between habitats (ANOVA: biovolume: $F_{2,14} = 4.2$, $p = 0.038$; cell number: $F_{2,14} = 0.8$, $p = 0.488$; cell size: $F_{2,14} = 5.0$, $p = 0.023$; species richness: $F_{2,14} = 8.8$, $p = 0.003$; Fig. 3). However, contrary to the expectations from the NMDS plot, there were also no significant differences between the habitats for the organic matter or chlorophyll-*a* content of the sediment (ANOVA: chlorophyll-*a*: $F_{2,14} = 1.9$, $p = 0.187$; organic matter content: $F_{2,14} = 1.6$, $p = 0.233$; Fig. 3).

Fig. 1 Experimental effects on diatom community composition and properties of the biofilm by adding small artificial mussel reefs to an intertidal flat (comparing reef vs bare flat), in an exposed sandy habitat (sand) and a more sheltered wake of a blue mussel reef (wake). **a** biovolume of diatoms, **b** number of diatom cells, **c** mean biovolume of the individual cells, **d** species richness of the diatom community, and **e** the organic matter content of the upper 5 cm of the sediment. Error bars show standard error of the mean; in **a** and **b** the bars show SE for the total biovolume and the sum of all diatom cells, respectively. The legend on the top rank the species left to right from smallest to largest size. For abbreviations of species names, see Table A.4.4.

DISCUSSION

We show that mussel reefs can change species composition and the size distribution of the biofilm by promoting higher numbers of small motile diatoms. Constructing small mussel reefs increased diatom cell numbers and decreased the cell size, and this was caused by a strong increase in abundance of a few small species. The field study was sampled later in the season with lower general production and abundance. Still, the general decrease in cell size was apparent when comparing the front to the wake of the mussel reef, and this was caused by a change in species composition where a small diatom species (*Navicula* spp.) was more common in the wake of the reef, and one of the largest diatom species in the area (*Pleurosigma aestuarii*) prevailed in front of the mussel reef. At the same time, species richness and chlorophyll-*a* concentrations in the biofilm in general correlated positively with the species composition of diatoms around the mussel reef. The size of diatoms determines growth and gross photosynthesis rates where smaller cells have a higher productivity (Geider et al 1986; Morin et al 2008), and earlier published results showed that the artificial mussel reefs increase chlorophyll-*a* concentrations in the sediment (Engel et al 2017). Thus, the small reefs mimicked and reinforced parts of the natural pattern in diatom community composition and function that we documented in the field, indicating that the natural intertidal shellfish reef has a significant impact on the function of the biofilm in the study area. The function of the intertidal biofilm provides large ecosystem services by stabilising the surface of coastal sediments and increasing sedimentation (Grant et al 1986; Vos et al 1988; Paterson 1989; Hoagland et al 1993; Malarkey et al 2015); improving water quality by binding toxic metals and pathogens (Decho 2000); contributing to coastal production (Colijn and de Jonge 1984) and sustaining the intertidal food-web (Christiaan et al 2017). This highlights the importance of intertidal shellfish reefs for intertidal ecosystems, supporting ecosystem functions and services for coastal populations by promoting the biofilm (Widdows and Brinsley 2002; van der Zee et al 2012; Engel et al 2017; Eriksson et al 2017).

Fig. 2 Nonmetric Multidimensional Scaling (NMDS) results showing the **a** site and **b** species scores for the two first ordination axes. The ellipses are ellipsoid hulls that enclose all site scores of each habitat type (green: sand, red: wake of mussel reef, grey: front of mussel reef). The arrows show significant correlations with environmental and biological variables ($p < 0.05$). Abbreviations arrows: size average size of cells in the sample, biovol total biovolume of the sample, chlorophyll-a chlorophyll-a content in the top 2 mm of sediment, OM_5 organic matter content of top 5 cm of sediment, sp. rich number of species in the sample. The species scores are the weighted average of the site scores for each species along the ordination axes. The weighted average is constructed by weighing the biovolume of each species to corresponding value on the NMDS axes for each site. For abbreviations of species names, see Table A.4.4.

The experimental reefs were very small compared to natural reefs, and it is not clear how the small-scale experimental effects compare to the natural large-scale effects that mussel reefs have on their surroundings. Natural mussel reefs stabilise the sediment up to 100 m in the wake of their physical structures (Donadi et al 2013a, b; van de Koppel et al 2015). However, the species composition changed correspondingly when comparing small- and large-scale effects in the experiment: the artificial mussel reefs promoted smaller species, and the species were smaller in the wake of the mussel reef than on the sandy plain. Thus, the small-scale effect of the artificial mussel reef was similar to the large-scale effect of the natural mussel reef. The diatom community was, in general, dominated by small *Navicula* species, which is common for intertidal sediments in the Wadden Sea (Janssen et al 1999). The sheltered wake of the mussel reef with less sediment erosion and more cohesive sediment was characterised by

smaller species of diatoms (length < 10 μm) (Sabbe 1997), such as *Nitzschia* spp., *Cylindrotheca closterium*, and the smallest *Navicula* species.

Fig. 3 Composition of motile diatom species in samples collected from a sand flat, the wake of a mussel reef and the front of the same mussel reef, based on **a** biovolume, **b** number of individuals, **c** mean biovolume of the individual cells, **d** species richness of the diatom community, **e** chlorophyll-a concentrations in the upper 2 mm of the sediment, and **f** the organic matter content of the upper 5 cm of the sediment. Error bars show standard error of the mean; in **a** and **b** the bars show SE for the total biovolume and the sum of all diatom cells, respectively. The legend on the top rank the species left to right from smallest to largest size. For abbreviations of species names, see Table A.4.4.

The sandy area, with high sediment erosion and more non-cohesive sediment, and the front of the mussel bed with strong hydrodynamic conditions, was characterised by larger species (mainly epipelton: length of $> 10 \mu\text{m}$), such as *Pleurosigma aestuarii*, *Entomoneis paludosa*, *Stauroneis* spp., and the largest species of *Navicula*. Earlier, field studies have highlighted the importance of sediment erosion for diatom community composition, suggesting that the adhesion capacity of the diatoms themselves and the sediment sorting (grain size) by hydrodynamic conditions determine the diatom assemblage structure (e.g. Colijn and Dijkema 1981; Sundbäck 1983; De Jonge 1985; Sabbe and Vyverman 1991; Ribeiro et al 2013). Diatom cell size affects attachment capacity, and exposure to wave action determines the size distribution of periphyton diatom communities on hard substrates (Busse and Snoeijis 2003). Accordingly, Delgado et al (1991) showed that large diatom species were favoured in exposed sandy substrates with strong hydrodynamic conditions, suggesting they were less vulnerable in a harsh sedimentary environment. Our study indicates that the physical protection against wave exposure provided by the formation of shellfish reefs creates a habitat favouring small motile benthic diatoms sensitive to sediment erosion. The vertical migrating behaviour of the pennate diatom community that moves around in the top layer of the sediment is characteristic for intertidal flats (Colijn and De Jonge 1984; de Jonge 1985). The migrating behaviour is also important for the development of the microphytobenthos biofilm. Motility is linked to the occurrence of extracellular polymeric substances (EPS), which is exuded from their raphe, a slit in their silicate exterior (frustule) (Lind et al 1997). The migration of diatoms is strongly promoted by light, and increasing light leads to an accumulation of diatom cells on the sediment surface (Sauer et al 2002). The EPS exuded by migratory diatoms contributes significantly to the biofilm matrix (Stal and Brouwer 2003). There is a negative correlation between grain size and the amount of EPS in the sediment (Brouwer et al 2003), and diatom biomass is, in general, higher in cohesive than in non-cohesive sediment (in cohesive sediment, the grain size diameter is less than $63 \mu\text{m}$) (De Brouwer and Stal 2001; Kromkamp and Foster 2006); indicating that biofilms probably can only

develop in sediment characterised by low energy (Stal and Brouwer 2003). This further supports the hypothesis that it is the sediment stabilising effect that enhanced the biofilm development on the artificial and natural shellfish reefs in our study, shown by an increasing number of diatom cells or biovolume in the sediment. The interaction between the biofilm and shellfish reefs indicates the importance of facilitative processes between individual organisms and organism groups in harsh environments with high abiotic stress, such as tidal flats (Maestre et al 2009; Eriksson et al 2010). By ameliorating hydrodynamic disturbance, blue mussel reefs facilitate the biofilm and thereby create regions with high microphytobenthos biomass and primary production (Donadi et al 2013a, b; Engel et al 2017; Eriksson et al 2017, this paper). At the same time, because of resuspension, the microphytobenthos play a major part in benthic–pelagic coupling and contribute strongly to the production in estuaries and shallow coasts (Underwood and Kromkamp 1999). As such, the biofilm is important both for deposit and suspension feeding organisms (Jonge and Beusekom 1995; Ubertini et al 2012), and an important food-source for the blue mussel reefs. Thus, our results strongly suggest that by initiating small-scale diatom sediment feedbacks, intertidal blue mussel reefs create areas of high production and biodiversity that they themselves are favoured by, potentially promoting also larger-scale self-reinforcing feedbacks.

ACKNOWLEDGEMENTS

We thank Vereniging Natuurmonumenten and the Province of Friesland for granting us permission for field work on the tidal flats; Tjeerd Bouma at NIOZ for lending us his specially made Shear Vane for intertidal sediments; Javier Alegria, Luna van der Loos and Just van der Endt for their help in setting up the field experiment; and Stella Bos for help in the laboratory. This work was supported through a scholarship of the Indonesia Endowment Fund for Education (LPDP) to R. Andriana.

Appendix A4.1

Sampling layout and location of experimental sites

Figure A4.1 Map of the four sampling transects and the two experimental sites. The raster show the location of the shellfish reef. The green plusses show sampling points more than 75 m away from the mussel bed on sandier sediment (Bare sand); red triangles show sampling points less than 50 m away on the shoreside of the shellfish reef (Reef wake) in muddy sediment; grey circles show sampling points less than 50 m away on the seaside of the shellfish reef (Reef front). The grey larger rectangles show the location of the two experimental sites.

Appendix 4.2 Testing “fence” effects

We analysed the effects of having a rope or a plastic fence (“fence” effects) around the plots for all response variables by comparing having no fence with having different types of fences in the plots without mussel additions. Thus, we compared the “no mussel additions and no fence” treatment (the unfenced control) with the “bare flat and plastic fence” and “bare flat and rope fence” treatments, respectively. We analysed “fence” effect on species composition based on both biovolume and cell numbers, using permutational multivariate analysis of variance (PERMANOVA) (analysing distance matrices using the `adonis2` function in the `vegan` package `vegan` package, version 2.5-6; R Core Team, 2020; Oksanen et al. 2019). We analysed “fence” effect on total biovolume, total cell number, average cell size and species richness of the diatom community, as well as organic matter content of the top 5 cm of the sediment; by fitting a linear model (R version 3.6.1). This enabled us to use the `summary` command to get post-hoc contrasts, comparing the rope and the plastic fence separately against the unfenced control. All models contained the fixed explanatory factor type of fence (plastic net, rope or unfenced control).

There were no significant effects of the ropes or the plastic fence on any of the response variables, compared to the unfenced control. There was no significant difference in diatom species composition between the plots with different types of fence (rope, plastic, or no fence), not for the multivariate analyses based on biovolume or cell numbers (PERMANOVA; biovolume: $F_{2,13}=1.5$, $p=0.173$; cell number: $F_{2,13}=1.5$, $p=0.168$). We also did not detect any significant “type of fence” effects on total biovolume, total cell number, average cell size or organic matter content of the top 5 cm of the sediment (Table A.4.2; Fig. A.4.2). There was a significant “type of fence” effect on diatom species richness (Table A.4.2).

However, this effect depended on a difference between the plots with a rope and plastic fence, while none of the types of fences showed a significant different species richness

Appendix 4.2 (continued)

compared to the unfenced control (rope fence vs unfenced control: $t=-1.3$, $p=0.217$; plastic fence vs unfenced control: $t=1.8$, $p=0.106$; Fig. A.4.2d).

Table A.4.2 *Linear model results comparing effects of having different types of fence around the plots: a rope fence, a plastic fence or an unfenced control.*

	df	F	p
total numbers of diatom cells	2,13	1.0	0.405
total biovolume of diatoms	2,13	1.2	0.334
average cell size of diatoms	2,13	0.1	0.904
diatom species richness	2,13	4.2	0.039
organic matter content (5 cm)	2,13	0.213	0.811

Appendix 4.2 (continued)

Fig. A.4.2 Procedural experiment effects on properties of the biofilm by adding different types of fencing around the plots (“fence” effects). a) biovolume of diatoms, b) number of diatom cells, c) mean biovolume of the individual cells, d) species richness of the diatom community, and e) the organic matter content of the upper 5 cm of the sediment. Error bars show 0.95 % confidence intervals.

Appendix 4.3 Model selection based on Akaike's Information Criteria, corrected for small sample size (AICc)

AICc values for univariate models testing the effects of mussel additions (mussel additions or not), different types of fencing (plastic fence or rope) and site (exposed sand plain or more sheltered wake of mussel bed); on total biovolume of diatoms, total cell numbers, average size of diatom cells in sample, diatom species richness and the organic matter content of the upper 5 cm of the sediment. The candidate models were analysed using ANOVA for unbalanced designs (Type III ANOVA using the car package in R, Version 3.0-10) and included different levels of interactions (full factorial model: site*fence*mussel addition; two-way factorial model: site + fence + mussel addition + site:fence + fence:mussel addition + site:mussel addition; main effects model: site + fence + mussel addition). AICc estimates the quality of a model, relative to the other models. The best model according to AICc is indicated in bold.

Table A.4.3 AICc values for 1) full factorial, 2) two-way factorial and 3) main effects model

Candidate model:	full factorial model	two-way interaction model	no interactions model
total numbers of diatom cells	85.38	81.69	69.13
total biovolume of diatoms	28.19	23.02	12.03
average cell size of diatoms	-9.89	-14.44	-21.83
diatom species richness	95.43	91.55	94.93
organic matter content (5 cm)	-4.84	-10.33	-17.3

Appendix 4.4 Biovolume of microalgae species and species groups found in the study

Table A.4.4 Diatom species and species groups based on size found in the field study on the intertidal flat south of Schiermonnikoog, the Netherlands.

Species	Abbreviation	Average biovolume in sample ($\mu\text{m}^3/\text{ml}$)
<i>Amphora coffaeiformes</i>	AMF	122.37
<i>Amphora</i> sp.	AMP	797.93
<i>Cylindrotheca coelestium</i>	CYL	38.95
<i>Entomoneis paludosa</i>	ENT	4322.52
<i>Gyrosigma acuminatum</i>	GYA	1440.38
<i>Gyrosigma fasciola</i>	GYF	212.38
<i>Hantzschia</i> sp. (medium)	HAN_II	619.39
<i>Navicula forcipata</i>	NAF	1586.33
<i>Navicula</i> sp. XS (extra small)	NAV_I	8.06
<i>Navicula</i> sp. S (small)	NAV_II	50.11
<i>Navicula</i> sp. M (medium)	NAV_III	148.16
<i>Navicula</i> sp. L (large)	NAV_IV	735.1
<i>Nitzschia sigma</i>	NITZ_I	192.93
<i>Nitzschia</i> sp. II (small)	NITZ_II	38.11
<i>Nitzschia</i> sp. III (medium)	NITZ_III	203.22
<i>Nitzschia</i> sp. IV (large)	NITZ_IV	359.75
<i>Petroneis humerosa</i>	PET	2844.88
<i>Pinnularia</i> sp.	PIN	2370.28
<i>Pleurosigma aestuarii</i>	PLE	4813.84
<i>Stauroneis</i> sp. (small)	STA_I	472.36
<i>Stauroneis</i> sp. (medium)	STA_II	1917.41
<i>Stauroneis</i> sp. (large)	STA_III	10821.6

Appendix 4.5 ANOVA main effects results of mussel additions, different types of fencing and site on properties of the biofilm

Results from the ANOVA model selected by AICc to have the highest quality (see Table A.4.3); describing effects of mussel additions (mussel additions or not), different types of fencing (plastic fence or rope) and site (exposed sand plain or more sheltered wake of mussel bed) for each of the response variables: total biovolume of diatoms, total cell numbers, average size of diatom cells in sample, diatom species richness and the organic matter content of the upper 5 cm of the sediment (ANOVA for unbalanced designs; Type III ANOVA using the car package in R, Version 3.0-10). Note that for species richness, the main effects are extracted from the two-way factorial model. For all other response variables, the effects are from the main effects model (no interactions included in the model).

Table A.4.5 ANOVA results of different types of, different types of fencing, mussel additions, and site

	fence type		mussel addition		site	
	F	p	F	p	F	p
total numbers of diatom cells	2.2	0.156	4.6	0.046	17.1	<0.001
total biovolume of diatoms	1.7	0.205	0.7	0.417	11.1	0.004
organic matter content (5 cm)	0	0.838	0.1	0.768	36.7	<0.001
average cell size of diatoms	0.1	0.825	7.4	0.014	0.8	0.373
diatom species richness	8.5	0.011	0.7	0.434	30.7	<0.001

Note: df fence = 1, df mussel addition = 1, df site = 1, df error = 18

#the beauty of life_benthic diatoms (©R. Andriana)

The power of question is the basis of all human progress.
-Indira Gandhi-

CHAPTER 5

Preliminary study :

Molecular identification of benthic diatom isolated from the tidal flats of Schiermonnikoog by applying *rbcL* as the genetic marker

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ABSTRACT

The composition of benthic diatom communities contributes strongly to ecosystem function. However, their taxonomical identification is demanding, making ecological studies of diatom community composition challenging. Here, we carried out a molecular approach to species identification by using the potential chloroplast genetic marker - *rbcL* gene. We prepared the materials for molecular analysis by isolating and culturing benthic diatoms species using environmental samples collected from an intertidal flat. We then proceeded with molecular analysis: DNA purification, PCR amplification targeting the *rbcL* gene, Sanger sequencing and sequence identification by applying BLAST through NCBI's Genbank. The *rbcL* genes from 5 monoculture benthic diatom samples were sequenced successfully with high sequence identities (98 -100%) and supported by their phylogenic-likelihood tree (bootstrap values > 80%). These results confirmed the manual counts which indicating the dominant benthic genera on the intertidal mudflat is *Navicula*. In this study, we resolved that the molecular approach likely can contribute significantly to rapid high throughput species identification of diatoms. The success of the method relies substantially on the quality of starting material and is currently limited by the quality of database references.

INTRODUCTION

Benthic diatoms, mobile and attached, are autotroph unicellular organisms that inhabit tidal flat and contribute significantly to the total primary production in estuaries and tidal flats (Reise, 1985; Kromkamp et al., 2006; Christianen et al. 2017). They stabilize the sediment by producing biofilm (Stal and de Brower, 2003), contribute in global carbon sinks of the coastal zone (Guarini, et al., 2008) and practice as bioindicators in assessing water quality (Desrosiers, et al., 2013). Furthermore, in shell-reef reefs on tidal flats, they facilitate other organisms and create hot spots of production and biodiversity on tidal flats (Engel, et al., 2017). We find them abundantly in temperate intertidal areas, where they display a complex community dynamic with seasonal succession (Admiraal, et al., 1984) and self-organized spatial patterns (Weerman et al., 2012).

Accurate diatom identification is highly demanding, making research into the function of diatom community composition difficult. The traditional approach use microscope, which is very time consuming and require a high level of taxonomical knowledge; the small sizes of diatoms, unclear definition and indefinite differences make identification to the species or even genus level intricate (Morales, et al., 2001, Zimmermann, et al. 2014). However, molecular technology using DNA sequencing has the possibility to be rapid and generally significant depending on the quality of the DNA barcodes library (Rach et al., 2008). Kaczmarek et. al. (2007) combined morphological, molecular and life history features to analyze their compatibility in diatom taxonomy. Further, Witkowski et. al. (2020) explored both methods to reveal the clarity of the forms and genera of Bacillariophyta (using marine diatoms). Still, the use of molecular approach for diatom research is limited by the identification of the best genetic marker and an often-incomplete database of species-specific sequences (Mann, et al., 2010; Sung, et al., 2017).

Several researchers have addressed the issues of using molecular methods for determining the species level in the Bacillariophyta. Mann et al. (2010) suggested that the large subunit of the ribosomal gene (LSU rRNA) and ribulose biphosphate carboxylase large subunit coding gene (*rbcL*), which is a chloroplast gene, were potential genetic markers to implement in DNA barcoding of diatoms. Zimmermann et al. (2014) applied the V4 region of the 18S rRNA and *rbcL* genes, to sequence their strains in conducting a best practice in taxonomy reference libraries for environmental barcoding of diatoms. Guo et al (2015) tested the effectiveness of different genetic markers; 18S rRNA gene and the internal transcribed spacer (ITS) region, *mitochondrial cytochrome oxidase subunit 1* (COI) and *rbcL*. Finally, Sung et al (2017) compared 18S rRNA and *rbcL* genes. The results showed that the *rbcL* was more divergent than the 18S rRNA gene, leading to the conclusion that the *rbcL* gene was the most suitable genetic marker for diatom species identification (Sung et al, 2017).

Here, we therefore tested *rbcL* as a potential genetic marker for identifying benthic diatoms. We started with designing the protocols of culturing and isolating benthic diatoms before subjecting them to molecular analysis. We selected sampling areas based on several ecological studies that have been conducted on the tidal flats of Schiermonnikoog (Donadi et al., 2013a; 2013b; Engel et al, 2017; Eriksson et al, 2017). Regardless of technical problems with culturing pure strains of benthic diatoms from the sediment, we were able to isolate species and establish the suitable protocols for their molecular identification, including DNA extraction, PCR amplification and sequencing. Despite small amounts of DNA material and a limited *rbcL* public database, our results revealed high percentage of sequence identities, highlighting the potential of this approach to improve further studies on the diversity of benthic diatoms.

MATERIALS AND METHODS

Sample collections

We collected biofilm samples from two sites dominated by cockle beds on the intertidal mudflat south of the island Schiermonnikoog, the Netherlands, to obtain monocultures of motile benthic diatoms (hereafter referred to as “benthic diatoms”). The areas were located on the coast side of large mussel beds, on the east (N 53.47258°, E 6.21503°) and west side (N 53.46878°, E 6.18718°) of a ferry pier and the sampling collection was conducted during low tide.

At each site, we sampled the biofilm (three replicates per site) by scraping off the sediment surface, which contained a mixed (natural) diatom community. The samples were kept on falcon yellow-cap tubes of 15ml and transported on refrigerated condition back to the laboratory (<24h) for further treatment. In the lab, within 24h of the collection day, we began the process of isolation and culturing of single species of benthic diatoms (see below).

Monocultures sample isolation and culturing

As a preliminary study, we developed a protocol for culturing unicellular benthic diatom from the biofilm. First step was the isolation of single diatom strains on agar plates. Agar medium (1%) was fixed in a plate (Ø:10cm) with sterilized seawater ($\pm 30\%$, with a Sartorius Sartopore 2 150 filter) and autoclaved. After this, a small amount of biofilm sample was placed in the middle of the drawn grids ($n = 10$ squares) and added with very low nutrient concentrations (to a final concentration of N:P:Si, 83×10^{-2} : 16×10^{-4} : 92×10^{-3} mM). We placed all the agar plates in the climate chambers with air temperature of 19 to 25 °C to let them grow; the first observation took after approx. 7 days.

Fig. 1 A developed protocol of culturing unicellular benthic diatom from the biofilm: the isolation of single diatom strains on agar plate.

The growth of the benthic diatoms was observed under a Zeiss dissecting microscope indicating all grids where the diatom sample had moved away from the starting point. If the diatoms had moved conspicuously, the material in that grid was transferred into new agar plates with 4 grids separation for further growth. This transferring process was repeated until we obtained a clean monoculture, thus, an aggregation consisting of a strain developed from one original cell or population from a single species.

After getting monoculture of different species of benthic diatoms, the culturing process started by transferring them into 60-mL- culture flasks (TPP, filter screw cap) with the

same sterilized water as above as the culture medium. For slow-growing monocultures, we added extra nutrients of Provasoli to 1x strength (20mL of Provasoli in a liter of seawater).

Molecular analysis:

Sample selection

To select samples for molecular analysis, we first made a visual inspection of the monocultures. We selected eight cultures which had a good growth, notified by the thickness of the brown layer on the bottom of the culture bottle, and then examined them under the microscope (Table 1).

DNA extraction

DNA was extracted from both types of benthic diatom samples (monocultures and the biofilm mix-community samples) ($\pm 2\text{mL}$), by modifying the DNA extraction protocols from Kooistra (2002). Briefly, the samples were treated with 800 μL TE buffer and 2 μL of β -mercaptoethanol in the thermoblock (at 60°C) for 15 min. After this, the incubation continued at room temperature for 45 min with occasionally mixing using a rotor. When the incubation was ready, the mixture was shaken gently, complemented with an equal volume of chloroform-isoamyl alcohol [CIA; 24/1 (v/v)], vortexed and centrifuged at maximum speed for 10 min. The aqueous layer was collected and transferred it into the new Eppendorf tube and the CIA treatment was repeated (as described previously) then centrifuged for 15 min at maximum speed. The DNA in the aqueous phase was precipitated using cold iso-propanol (2/3 vol), mixed well and stored at 4°C for 1h; and then centrifuged for 30min at maximum speed. The pellet was washed with 100 μL of cold 80% ethanol, soaked for 15 min at -20°C and centrifuged. After centrifugation as much ethanol as possible was removed with a pipette. Last, the pellet was dried for about an hour and re-suspended in 50 μL of sterile milliQ water. We

determined the quantity of the DNA using NanoDrop (Appendix 5.1); and the quality using agarose gel electrophoresis (Appendix 5.2).

PCR amplification

PCR amplification was performed using *rbcL* gene; this primer selection was based on an earlier study of Sung et al (2017) that identified isolated benthic diatoms from a tidal flat, comparing morphological and molecular methods. The primer sets were DPrbc11 (5'-AAGGAGAAATHAATGTCT-3') and DPrbcL7 (5'-AARCAACCTTGTGTAAGTCTC-3'), which amplifies a fragment of 1,600 bp and 1,550 bp, approximately. We also modified and optimized the PCR reaction based on their protocols: the initial denaturation was at 95°C for 3min, followed by 35 cycles of main amplification (95°C for 3sec, 55°C for 1min 30sec, 72°C for 1min 30sec), with final extension at 72°C for 10min. PCR products of expected length (verified in agarose gels) were purified using a QIA quick PCR purification kit (cat. Nos. 28104 and 28106, Hilden, Germany) then sent to GATC Biotech, a German company specialist in molecular analysis for academic and industrial purposes, which applied Sanger sequencing method.

Sequencing alignment and phylogenetic analyses

We visually examined the quality of the sequence electropherograms and manually edited using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA); removing the low-quality bases at both ends. For further phylogenetic analysis, *rbcL* gene sequences were aligned with the GeneBank (www.ncbi.nlm.nih.gov) to determine the species identity by applying Basic Local Alignment Search Tool (BLAST). Two Ochrophyta species, *Nannochloropsis salina* D.J. Hibberd and *Ochromonas Danica* E.G. Pringsheim were used as outgroups. The multiple sequences alignment was performed using MUSCLE (Edgar, 2004) with default settings and was visually

corrected using Geneious 8.1.9 (Kearse, 2012). We applied the PhyML plugin in Geneious to a reconstruct Maximum-likelihood (ML) tree, using a GTR model, and estimated the gamma value and the proportion of invariable sites, all other options were set as default. The tree was rooted with the Ochrophyta outgroup sequences. The resulting tree was visualized using Fig Tree (Rambaut and Drummond, 2012) and node support was estimated using 1000 bootstrap replicates.

Comparing the methods

We manually examined the benthic diatom species in each of the monoculture samples under light microscope (LM) (400x and 10^3 x magnification). Taxonomy was determined to species or genus level, using the identification species list of diatoms in the Wadden Sea (pers com Engel, F., 2018); each species of benthic diatom in the samples were also documented with photos. Finally, we compared the BLAST' results with our microscope identifications.

RESULTS

Monoculture benthic diatom

The quantity and quality of nucleic acid concentration as determined by DNA purification, quantification and PCR products (Table 1 and 2; Fig. 1-3), was good enough to proceed with sequencing for all eight monocultures of benthic diatoms. The culturing of monocultures 11 and 46 was unsuccessful, they grew fast in a massive colony and collapsed when reaching the maximum growth. Still, we were able to extract enough high-quality DNA. However, two monocultures (16 and 50A) needed to be deleted due to handling error, leaving only six monocultures for sequencing (ID: A, AD, I, W, 11 and 46).

Table 1. NanoDrop DNA quantification of monoculture samples of benthic diatoms (μL): to determine the DNA volume of each sample to put in the gel wells of agarose gel electrophoresis. Due to handling error during DNA purification process, sample ID 16 and 50A were omitted from further analysis (written in cursive and labelled with #).

Identity (ID)	Nucleic acid concentration	The absorption wavelength of 260/280	The absorption wavelength of 260/230
A	104.7	1.96	1.86
AD	302.7	1.65	1.02
I	13.6	1.62	0.99
W	23.1	1.66	1.30
11	24.6	1.75	0.96
16 #	11.6	1.52	0.91
46	10.8	1.60	0.85
50A #	13.0	1.66	0.93

Table 2. NanoDrop DNA quantification of the PCR purified products of monoculture samples (in duplicate; μL). The samples ID with higher nucleic acid concentration in their duplicates were sent out for sequencing (written in bold and labelled with #).

Identity (ID)	Nucleic acid concentration	The absorption wavelength of 260/280	The absorption wavelength of 260/230
A_1	6.9	2.85	3.02
A_2*	9.5	2.29	3.02
AD_1*	4.4	2.72	1.79
AD_2	4	2.09	0.47
I_1	4.8	2.37	2.08
I_2*	5.8	2.27	2.7
W_1	4	2.84	3.45
W_2*	4.4	2.58	3.3
11_1	11.6	2.24	2.48
11_2*	12.5	2.39	2.6
46_1*	3.3	3.11	2.08
46_2	2.7	8.77	2.72

Microscope identification

All strains were classified as pennate-raphid diatoms; they have elongated cell walls with bilateral symmetry and a raphe (a long-narrow opening along the bottom side which allows the motility on surface) (Gross, 2012). We could confirm that monoculture A was of the genus *Entomoneis*, and that cultures I and W were of the genus *Navicula* (Table 3). As for the molecular based identification, we could not identify monoculture AD - it was not listed in our reference. Due the unsuccessful culturing in our benthic diatom samples, we were unable to identify ID 11 and 46.

Table 3. Microscope identification of benthic diatom monoculture samples belonging to 2 families, Entomoneidaceae and Naviculaceae, and 1 unidentified species.

Identity (ID)	Microscope footage	Family classification	Genus classification	NOTES
A		Entomoneidaceae	<i>Entomoneis</i>	400x magnification; length of <30µm
AD		?	?	No record of this species in our reference list; 200x magnification, length of <61µm
I		Naviculaceae	<i>Navicula</i>	400x magnification and due to the technical difficulty, the picture was taken by a normal camera.
W		Naviculaceae	<i>Navicula</i>	400x magnification and due to the technical difficulty, the picture was taken by a normal camera.
11	-	-	-	a short time-fast growing species;
46	-	-	-	unable to make documentation.

Molecular-based identification

The *rbcL* genes from 5 out of 6 monoculture benthic diatom samples were sequenced successfully; sample ID's: A, I, W, 11 and 46 had a complete base pairs whereas sample AD had an insufficient one, *short reads* (Appendix 5.4). In reference to this, the BLASTn result of each *rbcL* sequence as given in Table 3, following the best matched species and sequenced identity in the database. The successful genetic results came out with high sequence identities, ranging from 98-100%, with strong acknowledgment as benthic diatom species. These results were further edited applying FinchTV and resulted as well in high sequence identities (99-100%) of benthic diatom species (Table 4). These sequenced results successfully identified two monocultures to the species (A: *Entomoneis paludosa*, and 11: *Cylindrotheca closterium*), assigned the genera *Navicula* spp. to three other cultures (I, W, and 46) but could not assign any diatom sequences to the 6th culture (AD) due to incomplete sequence result. These identification results confirm earlier manual counts (Engel, 2018), indicating that individuals of the genus *Navicula* dominate the intertidal mudflat.

In determining the phylogenetic relationship among the isolate monocultures, a phylogenetic tree based on *rbcL* gene was constructed from the 5 sufficient sequences (apart from sample AD) (Fig. 2). Here, the strains were set apart by following their taxonomic positions which based on distinctive morphological descriptions (Appendix 5.5). This result is supported by the high similarity of these diatom sequences results that showed high similarity (< 97%).

Table 4. The benthic diatom species identity according to *rbcL* gene sequence, with the closest relative found in GenBank using BLASTn, and as a correspondence: the edited BLASTn results using FinchTV

Identity (ID)	BLASTn			FinchTV		
	Species name	Strain no.	Ident. (%)	Species name	Strain no.	Ident. (%)
A	<i>Entomoneis paludosa</i>	-	99	<i>Entomoneis paludosa</i>	-	100
AD	ERROR	-	-	<i>Sphingobium baderi</i>	-	92
I	<i>Navicula</i> sp. SZCZCH703		98	<i>Navicula trivialis</i>	TA83	99
	<i>Navicula</i> cf. <i>salinarum</i>	TA402		<i>Navicula</i> cf. <i>salinarum</i>	TA402	
W	<i>Navicula</i> sp. SZCZCH703		99	<i>Navicula</i> sp. SZCZCH703		100
	<i>Navicula</i> cf. <i>salinarum</i>	TA407		<i>Navicula</i> cf. <i>salinarum</i>	TA407	
	<i>Navicula</i> cf. <i>salinarum</i>	TA402		<i>Navicula</i> cf. <i>salinarum</i>	TA402	
11	<i>Cylindrotheca closterium</i>	-	100	<i>Cylindrotheca closterium</i>	-	100
46	<i>Navicula tripunctata</i>	DO3	98	<i>Navicula tripunctata</i>	DO3	99
	<i>Navicula</i> sp. ArM0002	-	99	<i>Navicula</i> sp. SZCZCH703		99

Fig. 2 Phylogenetic tree estimated using *rbcL* gene of 5 culture strains. Bootstrap values determined by maximum-likelihood, are shown on the nodes; it supported by PhyML with all options default.

Discussion

We propose that the molecular approach is a valid alternative to traditional methods in identifying benthic diatoms. In this study, we were able to successfully identify five out of six monocultures to genus level; while one monoculture was unidentified. However, the unidentified monoculture, was also unidentified in our manual counts – indication that it is an uncommon species that is not well described. This highlight the main problem with the method; that the data quality in the reference database is the key to the success of any molecular based identification analysis (Zimmerman et al., 2014). We used the International Nucleotide Sequence Database Collaboration (INSDC) that sets off in connection with DDBJ, EMBL and GenBank. It requires to be frequently revised due to constantly development of biological identification; updating in relation to alpha taxonomy is necessary to build a compressive and accurate reference library.

We also suggest to implementing *rbcL* as the genetic marker; in our study, *rbcL* gene performed high similarity of sequence results and phylogenetically systemize benthic diatoms. Sung et al., (2017) already showed that most strains of *rbcL* gene were detached accordingly in their taxonomic positions in contrast to 18S rDNA phylogenies. The use of marker gene *rbcL* was also suggested earlier by Hamsher et al. (2011) as a genetic marker for species-level in diatoms, and an alternative to COI-5P. Further, Hamsher and Saunders (2014) revealed the universality character of *rbcL*-3P for a rapid identification of genetic species and its ability to distinguish the species that need further examination; in their study case, *Berkeleya* sp1. and sp.11 formed two *rbcL*-3P genetic groups whereas a single ITS2 cluster. Yet, both markers revealed a significant degree of variations in this group than in other species groups. Therefore, it was suggested for further examination, using culture material, to discover the cause of this pattern. Our results support that *rbcL* is a reliable marker for diatom species identification.

The analyses were problematic because of issues with culturing - there was not enough starting material in conducting extra replications of DNA extracting and not enough quantity of benthic diatom species monoculture to perform all manual counting's. However, interestingly enough, although counting was prohibited, we were able to extract a limited amount of DNA which generated successful assignment of diatom species/genera to the sample. This shows the potential strength of the molecular method.

The recommendation for future study in benthic diatom molecular analysis is to assure that sufficient amount of starting material is available both in culture and environmental samples, by increasing the number of culture plates/species and additional sampling points on the field. Culturing is an important step of refining the technique, to be able to add missing species to the reference database. In spite of the ecological values of benthic diatom community, the improvement of their taxonomical identification is urgently needed; to enable us to process many more samples at a higher pace, and explore ecological question in natural communities of diatoms. In this study, we concluded that molecular approach has the capacity to promote rapid and definitive identification. Yet, it relies substantially on the quality of starting material as well as the database references, which for now is incomplete.

Appendix 5.1 Protocol of Nanodrop Microvolume Quantification of Nucleic Acids

1. To start, clean the upper and lower optical surfaces of the spectrophotometer by wiping off both optical surfaces using sterile milliQ water and clean tissue paper.
2. Open the NanoDrop software and select the Nucleic Acid application. First, apply the blank measurement by dispensing 1 μ L of sterile milliQ water onto the lower optical surface then lowering the lever arm, select “blank” in the Nucleic Acid application and choose “the type of nucleic acid” that will be measured.
3. As the blank measurement is complete, clean both optical surfaces with a clean tissue paper.
4. Follow by 2 μ L of the sample measurement onto the lower optical surface then lowering the lever arm and select “measure”.
5. The software will automatically calculate the nucleic acid concentration in both purity wavelength ratio : 260/280 and 260/230 nm, with a respectively value of 1.8 and 2.0 (for DNA and RNA).
6. After the measurement is taken, clean both optical surfaces with a clean tissue paper.
7. Repeat the step no: 4-6 for the next measurement.

Appendix 5.2 Protocol of agarose gel electrophoresis qualification.

Appendix 5.3 Agarose gel electrophoresis of DNA quantification.

Fig. A.5.3.1. (as continuation of Table 1) Agarose gel electrophoresis DNA quantification: to observe the quality of their nucleic acid concentration. In comparison with NanoDrop results, the bands on gel wells no. 1 and 2 (ID: A and AD) were clearly to be seen whereas on gel wells no. 6,7 and 8 (ID: 16, 46 and 50A), they produced unformed bands. These results were consistent with the nucleic acid concentration stated on Table 1.

Appendix 5.3 (Continued)

(i)

1st comb of agarose gel

electrophoresis

Gel wells no.	Identity (ID)
1-2	A
3-4	AD
5-6	I
7-8	W

(ii)

2nd comb of agarose gel

electrophoresis

Gel wells no.	Identity (ID)
9-10	11
11-12	wells were broken
13-14	46

Fig. A.5.3. 2 (as continuation of Fig. A3.1) Agarose gel electrophoresis of the last optimization PCR products: to finalize the samples for sequencing. Two combs were used to run the gel electrophoresis and, in general, the bands were clearly seen.

Appendix 5.3 (Continued)

Fig. A.5.3.3 Agarose gel electrophoresis DNA quantification, to confirm the quality of the diatom cultures nucleic acid concentration. The bands clarity were consistent with the nucleic acid concentration as shown on Table 2.

Appendix 5.4 The sequence's results (FASTA) of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene

5.4.1. Benthic diatom samples

>Sample ID A_ *Entomoneis paludosa*

```
GGTGTAATCCCTTACGCTAAAATGGGTTACTGGGATGCTGCATACGCAGTAAAAA
GTACTGATGTTCTTGCTTTATTCCGTATCACACCACAACCAGGTGTAGATCCAGTA
GAAGCTGCTGCTGCAGTAGCTGGTGAGTCTTCAACTGCAACATGGACAGTTGTATG
GACTGATTTATTAACAGCTTGTGACCGTTACCGTGCTAAAGCTTACCGTGTAGATC
CAGTTCCAAACACAACCTGATCAATACTTCGCATTCATCGCATACGAATGTGATTTA
TTCGAAGAAGGTTCTTTAGCTAACTTAACAGCGTCTATCATCGGTAACGTATTTCGG
CTTCAAAGCGATCTCTGCATTACGTTTAGAAGATATGCGTATTCCTCACTCTTACTT
AAAAACATTCCAAGGTCCAGCAACTGGTATCATTGTAGAACGTGAGCGTTTAAAC
AAATATGGTATTCCATTATTAGGTGCTACAGTAAAACCTAAATTAGGTTTATCTGG
TAAAAACTATGGTCGTGTAGTATATGAAGGTTTAAAAGGTGGTTTAGACTTCTTAA
AAGATGATGAAAACATTAACCTACAACCATTTATGCGTTGGAGAGAGCGTTTCTTA
TACTGTATGGAAGGTATTAACCGTGCATCTGCATCAACAGGTGAACTAAAGGTTCT
TTACTTAAACATTACTGCTGGTACAATGGAAGAAGTTTACAAACGTGCTGAGTACG
CTAAATCAGTAGGTTCTGTAATCGTTATGATCGATTTAGTAATGGGTTACACAGCA
ATTCAATCAATTGCTCTTTGGGCTCGTGAAAACGACATGTTATTACACTTACACCG
TGCTGGTAACTCTACATATGCACGTCAAAAAGAATCACGGTATTAACCTCCGTGTTA
TCTGTAAATGGATGCGTATGTCAGGTGTAGATCATATCCACGCTGGTACAGTTGTA
GGTAAATTAGAAGGTGATCCTTTAATGATTAGAGGTTTCTACGACATTTTACGTGA
AACAACTTAGATGTTAACTTACCATACGGTATTTTCTTCGAAATGACATGGGCAA
GTTTACGTAAATGTATGCCTGTAGCTTCAGGTGGTATCCACTGNGGTCAAATGCAC
CAATTAGTT
```

>Sample ID AD_?

TCAGTGCATCGCGCACATATTCATG

>Sample ID I_ *Navicula* spp.

```
GTGACCGTTACGAATCTGGTGTAATCCCTTACGCTAAAATGGGTTACTGGGATGCT
TCATACGCAGTTAAACAAACTGATGTTCTTGCAATTATCCGTATTACACCACAACC
AGGTGTAGATCCAGTAGAAGCTGCTGCTGCTGTAGCTG
GTGAATCTTCTACTGCTACATGGACAGTTGTATGGACTGACTTATTAACAGCTTGT
GACCGTTACCGTGCTAAAGCTTACCGTGTAGATCCAGTTCCAAATACAACAGATGT
ATACTTCGCTTTTCATCGCATACGAATGTGATTTATTCCG
AAGAAGGTTCTTTAGCTAACTTAACAGCATCTATTATTGGTAACGTTTTTGGATTTA
AGGCAATTTCTGCTTTACGTTTAGAAGATATGCGTATTCCTCACTCATACTTAAAA
ACATTCCAAGGTCCAGCTACGGGTATCATTGTGGAAC
GTGAGCGTTTAAACAAATATGGTATTCCATTATTAGGTGCAACTGTAAAACCTAAA
TTAGGTTTATCTGGTAAAACTACGGTCGTGTAGTTTTTCGAAGGTTTAAAAGGTGG
TTTAGACTTCTTAAAAGATGATGAGAACATTAACCTCAC
```

AACCATTTCATGCGTTGGAGAGAACGTTTCTTATACTGTATGGAAGGTATTAACCGT
GCATCAGCAGCTACAGGTGAAGTTAAAGGTTTCATACTTAAACATTACTGCAGCTAC
TATGGAAGAAGTGTACAAACGTGCAGAGTATGCTAAAG
CAGTTGGTTCTATCATCGTTATGATCGATTTAGTAATGGGTTATACAGCAATTCAA
AGTATTGCTTTATGGGCTCGTGAAAATGACATGCTTTTACATTTACATCGTGCTGGT
AACTCAACTTACGCGCGTCAAAAGAACCACGGTATTA
ACTTCCGTGTTATCTGTAAATGGATGCGTATGTCTGGTGTAGATCATATCCACGCT
GGTACAGTTGTAGGTAAATTAGAAGGTGATCCTTTAATGATTAAAGGTTTCTACGA
TATTTTACGTCAAACAACTTAGATGTTAATTTACCTT
ACGGTATTTTCTTTGAAATGACTTGGGCTAGTTTACGTAAATGTATGCCTGTTGCAT
CNNNNGGAATTCATTGGGGGCCAATGGCACCAATTAATTCCTATTTAGGGGGAG
GATGNATANTTACAATTCGGTGGGAGGACAATTGGGNA
NNCNGGATGGTNTTCAAGCCGGGTGCAACTGGCTAACCGGGTTGCTTTNNA

>Sample ID W_ *Navicula* spp.

TGACCGTTACGAATCTGGTGTAAATCCCTTACGCTAAAATGGGTTACTGGGATGCTT
CATACGCAGTTAAACAAACTGATGTTTCTTGCATTATTCCGTATTACACCACAACCA
GGTGTAGATCCAGTAGAAGCTGCTGCTGCTGTAGCTGG
TGAATCTTCTACTGCTACATGGACAGTTGTATGGACTGACTTATTAACAGCTTGTG
ACCGTTACCGTGCTAAAGCTTACCGTGTAGATCCAGTTCCAAATACAACAGATGTA
TACTTCGCTTTCATCGCATACGAATGTGATTTATTCTGA
AGAAGGTTCTTTAGCTAACTTAACAGCGTCTATTATTGGTAACGTTTTTGGATTTAA
GGCAATTTCTGCTTTACGTTTAGAAGATATGCGTATTCCTCACTCATACTTAAAAA
CATTCCAAGGTCCAGCTACAGGTATCATTGTAGAACG
TGAACGTTTAAACAAATATGGTATTCCATTATTAGGTGCAACTGTAAACCTAAAT
TAGGTTTATCTGGTAAAACTACGGTCGTGTAGTTTTCGAAGGTTTAAAAGGTGGT
TTAGACTTCTTAAAAGATGATGAGAACATTAACCTACA
ACCATTTCATGCGTTGGAGAGAACGTTTCTTATACTGTATGGAAGGTATTAACCGTG
CATCAGCAGCTACAGGTGAAGTTAAAGGTTTCATACTTAAACATTACTGCAGCTACT
ATGGAAGAAGTGTACAAACGTGCAGAGTATGCTAAAGC
AGTTGGTTCTATCATCGTTATGATCGATTTAGTAATGGGTTATACAGCAATTCAA
GTATTGCTTTATGGGCTCGTGAAAATGACATGCTTTTACATTTACATCGTGCTGGTA
ACTCAACTTACGCACGTCAAAAGAACCATGGTATTAA
CTTCCGTGTTATCTGTAAATGGATGCGTATGTCTGGTGTAGATCATATTCACGCTG
GTACAGTTGTAGGTAAATTAGAAGGTGATCCTTTAATGATTAAAGGTTTCTACGAT
ATTTTACGTGAAACAACTTAGATGTTAACCTTACCCTT
ACGGTATTTTCTTCGAAATGACTTGGGGCTAGTTTACGTAAATGTAAGCCTGGTT
GC

> Sample ID 11_ *Cylindrotheca closterium*

TGACCGTTACGAATCTGGTGTAAATCCCTTACGCTAAAATGGGTTACTGGGATGCTT
CATACGCAGTAAAACTACTGATGTTTTAGCTTTATTCCGTATTACACCACAACCA
GGTGTAGATCCAGTAGAAGCTGCTGCTGCAGTAGCTGG
TGAATCTTCTACAGCAACTTGGACAGTTGTATGGACTGATTTATTAACAGCTTGTG
ACCGTTATCGTGCTAAAGCTTATCGTGTAGATCCAGTTCCAAATACACCCGATCAA
TATTTTGCATTTATTGCATATGAATGTGATTTATTGA

AGAAGGATCTTTAGCTAACTTAACAGCTTCGATTATTGGTAATGTTTTTGGATTTA
 AAGCGGTATCAGCTTTACGTTTAGAAGATATGCGTATCCCACATTCATACTTAAAA
 ACATTCCAAGGTCCTGCTACTGGTATTGTGGTAGAACG
 TGAACGTTTAAACAAATACGGTACACCATTATTAGGTGCAACAGTAAAACCAAAA
 TTAGGTTTATCTGGTAAAACTATGGTCGTGTAGTATTTGAAGGTTTGAAAGGTGG
 TTTAGACTTCTTAAAGATGATGAGAATATTAATTCACA
 ACCATTTATGCGTTGGAGAGAAAGATTCTTAAATTGTATGGAAGGTATTAATCGTG
 CATCAGCGGCTACTGGTGAAGTTAAAGGTTTCATACTTAAACGTTACTGCTGCGACA
 ATGGAAGAAGTATACAAACGTTTCAGAATATGCGAAAGA
 AGTAGGATCTATCGTTATCATGATCGATTTAGTTATGGGATATACGGCAATTCAAA
 GTATTGCTTTATGGGCTCGTGAAAATGATATGCTTTTACATTTACACCGTGCTGGTA
 ACTCAACTACGCACGTCAAAAAAATCATGGTATTAA
 CTCCCGTGTAAATTGTAAATGGATGCGTATGTCTGGTGTAGATCATATCCATGCTG
 GTACAGTTGTAGGTAAACTAGAGGGTGACCCGTTAATGATTAAAGGTTTTTATGAT
 ACTTTACGTTTAAACAACCTTTAGATGTTAATTTACCTTA
 TGGCTTATTCTTCGAGATGTCATGGGCTANTTTACGTCGTTGTATGCCAGTTGCTTC
 TGGGGNAATTCATTGGGGTTCAGAAGCACCAATTNNTTCATTATTTAGGGGGATGA
 AGAATTATTACAATTCGGGGGGGGNNNCAATTGGTCCA
 CCCGAANNNGGTNNCNAACCTGGGGGCTACCGCTAAACCGNTTNGCTTTTAAAAAC
 CAAGGGATTTAACCCTTAACAAAAGTGGCTGAANAACCTTTATTCCAAAATTTTG
 GCCCCAAAATCTNNNGAATGGNNNTAAAAANNTNNGG
 NNCCNN

>Sample ID 46 *Navicula* spp.

TGTAATTCCTTACGCTAAAATGGGTTACTGGGATGCTTCATATGCAGTTAAAACAA
 CTGATGTTTTAGCATTATTCCGTATTACACCACAACCAGGTGTAGATCCAGTAGAA
 GCAGCAGCTGCAGTAGCTGGTGAATCTTCTACAGCTAC
 ATGGACAGTTGTATGGACTGATTTATTAACAGCTTGTGATCGTTACCGTGCGAAAG
 CTTACCGTGTAGATCCAGTTCCAAATACAACAGATCAATACTTCGCTTTTCATCGCA
 TACGAATGTGATTTATTCTGAAGAAGGTTCTTTAGCTAA
 CTTAACAGCTTCTATCATTGGTAACGTTTTTGGTTTTAAAGCAATTTCTGCTTTACG
 TTTAGAAGATATGCGTATTCCGCACTCTTACTTAAAAACATTCCAAGGTCCAGCTA
 CTGGTATCATTTGTAGAACGTGAGCGTTTAAACAAATA
 TGGTATCCCTTTATTAGGTGCTACTGTAAAACCTAAATTAGGTTTATCTGGTAAAA
 ACTACGGTCGTGTAGTTTTTCGAAGGTTTAAAAGGTGGTTTAGACTTCTTAAAAGAT
 GATGAAAACATTAACCTCACAACCATTTCATGCGTTGGAG
 AGAACGTTTCTTATACTGTATGGAAGGTATTAACCGTGCATCAGCAGCTACAGGTG
 AAGTGAAAGGTTTCATACTTAAACGTTCACAGCAGCTACTATGGAAGAAGGTGTACAA
 AACGTGCTGAGTACG

5.4.2. The outgroup

> GenBank: AF015576.1 *Nannochloropsis salina*

ACACAATTAAAGATACTGATGTACTTGCATTATTCCGTATTACTCCACAACCAGGG
 GTTGATCCAATTGA

AGCCGCTGCTGCCATCGCTGGTGAATCTTCAACAGCTACATGGACTGTAGTATGGA
 CTGACTTATTAACA
 GCTTGTGATGTTTACAGAGCAAAAGCTTATAAAGTTGATTCAGTTCCAGGTACTAG
 CGACCAATTCTTCG
 GTTACGTTGCATATGAATGTGACCTTTTTGAGGAAGGTTCAATCGCTAACTTAACG
 GCTTCAATCATCGG
 TAACGTATTTGGATTCAAAGCTGTAAAAGCGTTACGTCTTGAAGATATGCGTATGC
 CTTATGCTTACTTA
 AAAACTTTCCAAGGTCCAGCTACTGGTGTGATGGTTGAACGTGAGCGTTNNNNNN
 NNNNNNNNNNNNNNN
 NNN
 NNNNNNNNNNNNNNNNNNN
 NNN
 NNNNNNNNNNNNNNNNNNN
 GAACGTTTCTCGTNNGTAATGGAAGGTGTTAACAGATCAGCAGCAGCTTCTGGTG
 AAGTTAAAGGTTCTT
 ACCTTAATGTTACAGCAGCCACTATGGAAGAAATGTACGAACGTGCTGAATTTGC
 AAAACTTATTGGTTC
 AGTAATTATCATGATCGACTTAGTGATTGGTTACACTGCAATTCAATCAATGGCAG
 TTTGGTCTCGTAAA
 AATGACATGATCCTTCACTTACACCGTGCAGGTAACCTCGGCATATGCTCGTCAAAA
 AAATCATGGTATTA
 ACTTCCGTGTAATTTGTAAATGGATGCGTATGGCTGGTGTGACCATATTACGCG
 GGTACTGTTGTAGG
 TAAACTAGAAGGTGATCCTTTAATGGTTAAAGGTTTCTATAATACATTATTGCAA
 CTAGTTTAGACATT
 AACTTACCACAAGGTATTTTCTTTGAACAGGACTGGGCTTCTTTAACAAAACTCT
 ACCTGTAGCCTCTG
 GTGGTATCCACTGTGCACAAATGCACCAATTACTTAACTACTTAGGTGNNNNNN
 NNNNNNNNNNNNNNGG
 TGGTGGTNNNNTTGGTCTCCCTGATGGATCGCTTTCTGGTGCAACAGCGAATCGTG
 TGGCTATGGAATGT
 GTACTTTTAGCTAAATACGAAGGTAAAGATTATATTAACGAAGGACCAAAAATCT
 TACGTGCTGCTGCAG
 AAAGCTGTGCTCCATTACGTACTGCTTTAGATCTTTGGAAAGATATTGCTTTCACT
 ATACATCAACAGA
 TACTGCAGATTACGTTGAAACAACAATAACAG

> **GenBank: GU935657.1_ *Ochromonas danica* strain SAG 933.7**

AAAGATACAGATATACTTTGCTTATTTTCGTGTAACCTCCACAACCTGGAGTAGATCC
 AATCGAAGCTGCTG
 CTGCTATTGCAGGAGAATCTTCTACAGCTACATGGACTGTTGTTTGGACGGATTTA
 TTAACAGCTTGTGA
 TATATACCGTGCTAAGGCTTACTTTGTAGAAGAAGTACCAAATAAACCAAATGAA
 TTTTTTGCTTTTATT
 GCATATGAATGTGATCTATTTGAAGAAGGTTCTATAGCAAATTTAACAGCTTCAAT
 TATTGGTAATGTAT

TTGGATTTAAAGCAGTAAAAGCTCTTCGTCTAGAAGATATGCGTCTTCCTTATGCT
TATTTAAAAACATT
TATTGGTCCTGCTTCTGGTGTGTTGTAGAACGTGAAAGATTAGATGTATTTGGAC
GTCCTCTTTTAGGT
GCAACAGTAAAACCTAAATTAGGTTTATCAGGAAAAAATTATGGACGTGTTGTTTA
TGAAGGATTTAAAG
GTGGACTTGATTTTTTAAAAGATGATGAAAATATAAATTCTCAACCATTCATGAGA
TGGCGTGAAAGATT
TTTATATGTTATGGAAGGTATTAACAAAGCATCAGCAAAGACAGGTGCTGTAAAA
GGTTCTTATCTTAAT
ATAACAGCAGCAACAATGGAAGATATGTATGAACGAGCAGAATTTGCAAGAGAA
ATAGGTTCTGTAGTTG
TAATGATTGATTTAGTTATTGGATATACAGCTATTCAAACAATTTGTCATTGGGGA
CGTAAAAATGATAT
GATTATTCATTTACATCGTGCAGGACATTCAACATATACTCGTCAAAAAAATCATG
GGATTAATTTCCGT
GTTATTTGTAAATGGATGAGAATGTCTGGAGTAGATCATATTCATGCAGGAAGTGT
TGTTGGAAAACTTG
AAGGTGATCCAAATACTGTAAAAGGATTTTATGATACACTTCTTTTACCAAGCTTA
AAAGAAAATCGTGG
ATTAGGTATTTCTTCGATATGGATTGGGCTTCTTTAAGAAAATGTATGCCAGTTGC
TTCTGGAGGAATA
CATTGTGGACAAATGCATCAA

Appendix 5.5 Light microscope images of benthic diatoms sequencing results

Fig. A.5.5 Light microscope images of diatoms sequenced in this study belonging to:
 (a) *Entomoneis paludosa*. (b) *Navicula* cf. *salinarum* TA402. (c) *Navicula trivialis* TA83.
 (d) *Navicula* cf. *salinarum* TA407. (e) *Cylindrotheca closterium*. Scale bar = 10 μ m.
 Note that scale bars of 9 and 16 are inside of the picture. (Sung, et.al., 2017)
<https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0179422>.
 (f) *Navicula tripunctata*. Scale bar 10 μ m. Potapova, M. (2009).
https://diatoms.org/species/navicula_tripunctata

Rinca island, Komodo National Park, East Nusa Tenggara, Indonesia
(©R. Andriana)

**#Ain't no mountain high enough.. ain't no valley low enough..
Ain't no river wide enough..
-Marvin Gaye & Tammi Terrell-**

CHAPTER 6

SYNTHESIS

In this thesis, I investigated the function of shellfish reefs for benthic algae in soft-sediment habitats. I specifically addressed the following research questions:

- Has the transformation of the native blue mussel reefs by the dramatic increase of the invasive Pacific oyster affected the development of benthic macroalgae on the now shared reefs?
- What is the ecological function of shellfish reefs for benthic primary production in soft-sediment intertidal ecosystems?
- How do mussel reefs affect the distribution of marine benthic diatoms?
- How to best apply a molecular approach in identifying marine benthic diatoms

I explored the research questions by performing a mix of ecological experiments and field studies. In the experiments I modified the habitat by creating small reefs of cockles, mussels and oysters and observed the effect of the different shellfish reefs on seaweed and microphytobenthic community composition and distribution (chapter 2, 3 and 4). In the field studies I analysed the relation between microphytobenthos and different environmental variables in transects that crossed a tidal flat with a natural blue mussel dominated reef (chapter 3 and 4). Finally, as an important contribution to enable benthic diatom studies that are interested in species composition and traits, chapter 5 present a basic protocol for how to identify benthic diatoms through molecular analysis; using *rbcL* gene as the selected genetic marker.

In this closing chapter, I discuss the main findings as well as the conclusions of each chapter and present my final concluding remarks.

The invasion of Pacific oysters influences the structure and function of intertidal shellfish reefs.

In chapter 2, I performed bio-manipulation experiments to test the effects of the Pacific oyster invasion of native blue mussel reefs, on seaweed communities. The past decades, an invasion of the Pacific oyster (*Crassostrea gigas* Thunberg, 1793; alternate representation *Magallana gigas*) has led to a dramatic transformation of intertidal shellfish reef communities across the Wadden Sea (Kochman et al. 2008, Reise et al. 2017a). The Pacific oyster overgrew and replaced the dominating native reef forming species, the blue mussel (*Mytilus edulis* L.), to form mixed oyster – mussel beds (Reise et al. 2017b). In my study area, pure blue mussel beds were intact as late as 2012, and have since then changed into mixed oyster – mussel beds in only ten years (personal observation).

Species specific algal communities with distinctly different properties developed on the different small-scale shellfish reefs treatments on the intertidal mudflat. Reefs constructed by native blue mussels developed a thick brown and structurally complex meadow of *Fucus vesiculosus* (bladderwrack). Reefs constructed by Pacific oysters developed a thin mat of *Ulva* spp. (sea lettuce) (Fig. 6.1).

Figure 6.1. Bio-manipulation (BM) shellfish reefs treatment: the development of seaweed community in one of BM treatment of (i) mussels and (ii) oyster reefs over the observation period. The graphs (iii) show that blue mussel reefs facilitated complex meadows of brown algae (*Fucus vesiculosus*) whereas Pacific oyster reefs facilitated green algal mats (dominated by the genus *Ulva*). Modified from Chapter 2; Andriana et al. 2020.

Another main finding in this study was revealed by an additional field survey. It showed that the presence of invasive Pacific oysters on a reef previously completely dominated by native blue mussel, significantly depleted the development of the *Fucus vesiculosus* meadow on the reef. Thus, the ecological complexity of this extremely important intertidal habitat, was decreased by the presence of the Pacific oysters. This highlights the ecological importance of byssus threads in intertidal ecosystems. The *F. vesiculosus* community develop on the reefs by means of entanglement of fragments and fully grown thalli in the byssus threads produced by the blue mussels (Albrecht and Reise 1994, Albrecht 1998, personal observation).

This study demonstrates that effects of the Pacific oyster invasion are not restricted to direct effects of competition amongst bivalves and associated invertebrates but also lead to cascading effects on an ecosystem level; due to differences in basic ecological functions of blue mussels and Pacific oysters.

Mussel beds increase the chlorophyll *a* content of the sediment and the primary production in the biofilm.

In chapter 3 and 4, I present the results from a bio-manipulation experiment and a field study, where I investigated how bivalve reefs influence and structure the microphytobenthic diatom communities that dominate the biofilm of intertidal sediments. In chapter 3 I focused on production by looking at effects on chlorophyll *a* and photosynthetic yield; in chapter 4 I focused on effects on the structure of the diatom community.

Chapter 3 present conclusive evidence that aggregations of mussels increase the local chlorophyll *a* concentration of shallow sediments on a tidal flat, and show that the primary production of the biofilm was elevated across a nearby natural mussel bed (Fig. 6.2). Chapter 3 also support the general long-range facilitative effect of mussel beds on its surrounding; by describing increased chlorophyll *a* concentrations in the sediment, and modified sediment conditions, up to 200m away from the mussel bed. Thus, chapter 3 demonstrate that mussel beds act as power stations that facilitate primary production of microphytobenthos on tidal flats.

Benthic microalgae are important primary producers in intertidal soft-sediment habitats that support the benthic food web (Underwood and Kromkamp 1999; Decho 2000; Stal 2003; Kromkamp et al. 2006; Markert et al. 2013; Rigolet et al. 2014). Chapter 3 highlight the importance of intertidal reefs in transforming and enriching the function of sedimentary systems by fuelling primary production, which in turn provides the energy base for a productive and diverse intertidal food web of invertebrates, fish and birds (Christiaan et al. 2017a).

Figure 6.2. Correlation between chlorophyll a concentration and photosynthetic yield (across the transects, for both year 2012 and 2016); Positive effect of mussel bed presence on both biomass and productivity of the benthic microalgae. From chapter 3; Engel et al. 2017.

Mussel beds control the composition and properties of benthic diatom communities by promoting higher numbers of small-sized cells and species.

Chapter 4 present conclusive evidence that blue-mussel beds influence the composition of motile diatom communities in the shallow intertidal sediment. A field study showed that the composition of the diatom community was significantly different in different types of habitats around an intertidal mussel bed; comparing the surrounding sandy flat, with the front and wake of the mussel reef (Fig. 6.3). First of all, the species richness of the diatom film was higher in both reef affected habitats (“reef front” and “reef wake”) compared to the sand flat. Second, *Navicula* spp. dominated the biofilm, but there were clear differences in sizes of the diatoms in the different habitats. The reef front was characterized by the largest *Navicula* cells, and other very large species

(*Pleurosigma aestuarii*, *Entomoneis paludosa*, *Stauroneis* spp.). The reef wake, on the other hand, was characterised by the smallest cells of *Navicula* and a high numbers of other smaller species (*Cylindrotheca closterium* and *Nitzschia* sp.). The sand communities were completely dominated by medium sized *Navicula* cells/species. Thus, the sheltered wake of the mussel reef, with less sediment erosion and more cohesive sediment, was characterised by smaller species; the sandy area with high sediment erosion and more non-cohesive sediment and the front of the mussel bed with strong hydrodynamic conditions was characterised by larger species (Fig. 6.3).

A field experiment provided conclusive evidence that the changes in diatom community composition across the natural reef was caused by the mussel bed itself. Small-scale reefs constructed in the intertidal mimicked and reinforced the natural pattern in diatom community composition and function that we documented in the field. The reefs changed the community composition of diatoms in the biofilm by promoting higher numbers of smaller sized cells and species. Small diatoms have higher growth and gross photosynthesis rates than larger, indicating that this explains the higher production and chlorophyll *a* concentration of the biofilm measured on the intertidal blue mussel reef in chapter 3.

Figure 6.3. The four sampling transects and the two experimental sites, with details description of bare sand, reef wake and reef front areas (i). The Nonmetric Multidimensional Scaling (NMDS) results showing site score for the two first ordination axes of each habitat type (ii) : the average size of the diatom cells and total biovolume was highest in the mussel reef front; the species richness of the diatom biofilm was higher in both reef affected habitats ('reef front' and 'reef wake'). Modified from chapter 4; Andriana et al. 2021.

A molecular approach can contribute significantly to rapid high throughput species identification of diatoms.

In chapter 5, I developed a protocol for molecular identification of benthic diatoms. Understanding what regulated the function of the biofilm in soft-sediment habitats, has traditionally been hampered by the time-consuming aspect of determining biovolume and species composition by counting and measuring cell sizes under the microscope. I therefore set out to test if metabarcoding is a technique that could be used to simplify the process of describing and understanding the function of diatom dominated biofilms.

The approach was partly successful. I sequenced the *rbcL* gene, which is a chloroplast genetic marker, and this resulted in high sequence identities for 5 out of 6 monocultures of benthic diatoms. Constructing a phylogenetic tree based on *rbcL* gene from the 5 supported sequences showed that the strains were set apart following their taxonomic positions based on morphological descriptions. The results confirmed the manual counts; indicating that the dominant benthic genera on the intertidal mudflat of Schiermonnikoog is *Navicula*.

The study shows that the molecular approach of metabarcoding likely can contribute significantly to rapid high-capacity species identification of diatoms. The protocol I developed generated high quality DNA, and the phylogenetic analyses of the sequences and manual inspection of the cells supported the results. However, the functionality of the method ultimately relies on the quality of starting material and is currently limited by the database references. In addition, the lack of reliable quantitative information means that metabarcoding is still a highly limited technique for ecological studies and monitoring of species communities.

Figure 6.4. Results of molecular approach in benthic diatom identification: microscope identification of the isolated benthic diatom (i), molecular identification according to rbcL gene sequence (ii) and the estimated phylogenetic tree-maximum-likelihood (iii). Modified from chapter 5.

DISCUSSION & CONCLUDING REMARKS

The benthic algae that I have researched in this thesis are the main primary producers of the Wadden Sea. *Fucus vesiculosus* meadows are the habitats with the highest density of primary biomass on the tidal flat (Baird et al. 2007), and the diversity of important foundation functions performed by *Fucus* canopies for associated organisms are well documented throughout the temperate world (e. g. Menge 1978, Jenkins et al. 1999b, Eriksson et al. 2006a, 2007). In the Wadden Sea, the function of mussel bed as biodiversity hot spots is well described; increasing the biodiversity of epifauna on the otherwise relatively bare tidal flat (Dittman 1990, Norling & Kautsky 2008, Markert et al. 2010, van der Zee 2012, 2015, van de Ouderaa et al. 2021a). Shellfish beds as bioengineers with an important function for marine biodiversity in general, has therefore become a central target for restoration and management measures in this soft sediment system. However, shellfish reefs with an associated *Fucus* meadow have a much higher biodiversity of associated invertebrate fauna than reefs without (van

der Ouderaa et al. 2021b). Still, there is remarkable few examples in the literature of research into the importance of *Fucus* meadows for the function of mussel reefs as biodiversity hot spots. Because of the invasion of Pacific oysters, the intertidal shellfish reefs have changed dramatically in the Wadden Sea the past decades; from a sea of mussels (Dankers & Fey-Hofstede 2015) to a sea of oyster reefs (mixed Pacific oyster – blue mussel reefs; Reise et al. 2017b). In chapter 2, I demonstrate that when the relative abundances of Pacific oysters increase and blue mussels decrease in the mixed shellfish reef matrix, the cover of the reef associated *Fucus* meadows is reduced. This suggest that the value for associated biodiversity of a Pacific oyster dominated reef is limited compared to a blue-mussel dominated reef. Considering the recent developments of programs targeting restoration of shellfish reefs in the Wadden Sea, it will be imperative to take into consideration the importance of *Fucus* meadows for the function of different types of reefs.

The microphytobenthos is the most important energy source for the benthic food-web in the Wadden Sea (Christianen et al. 2017a). The microphytobenthos forms a thin photosynthesising layer of biofilm that covers the sediment, where it contributes up to 50% of total primary production and support many other organisms (Decho 2000, Stal 2003, Kromkamp et al. 2006, Underwood and Kromkamp 1999, Markert et al. 2013, Rigolet et al. 2014). In addition, the microphytobenthos play a major part in benthic-pelagic coupling and thereby contribute strongly to the pelagic production in estuaries and shallow coasts (Underwood and Kromkamp 1999). In my thesis I demonstrate the fundamental importance of shellfish reefs in determining the structure and function of the biofilm. By ameliorating hydrodynamic stress, the physical structures of shellfish reefs change the composition and size distribution of the diatom community that construct and dominate the biofilm (chapter 4), and thereby increase the primary production and chlorophyll a content of the sediment (Donadi et al. 2013, Engel et al. 2017, Eriksson et al. 2017). This highlights the potential and importance of biological control of ecological processes in soft-sediment systems, where ecosystem

engineers can manipulate their own habitat and to a large extent improve their own living conditions; and in the process create local power stations that increase productivity and biodiversity (Eriksson et al. 2010).

The ability to construct, modify and enrich marine habitats is mostly described for prominent habitat forming ecosystem engineers such as seagrass meadows and shellfish reefs; which also forms the foundation for well described associated communities. However, although the biofilm consists of tiny organisms and do not protrude from the surface, it is a prominent ecosystem engineer in its own regard (Paterson et al. 1997). The biofilm manipulates the cohesiveness of the sediment by changing the property of the sediment surface; from individual grains that topple over each other at a certain shear stress, into an elastic membrane (Vignaga et al. 2013). The cohesiveness of the biofilm is created mainly by bacteria and diatoms that inhabit natural sediment, and which secrete extracellular polymeric substances (EPS). The EPS forms bonds between the sand grains and effectively glue them together (Decho 2000). This is called biostabilisation and result in that marine sediments are more difficult to erode with a biofilm than without (Paterson et al. 1997, Black et al. 2002); it can take up to five times as much force (shear stress) to break up and transport sediment that are covered by a biofilm (Grant & Gust 1987). Biostabilisation has an important function for bed formation, which controls sediment dynamics such as scour, erosion and deposition (Malarkey et al. 2015). Thus, the biofilm can modify the landscape on larger scales and decrease sediment transport (e.g. Weerman et al. 2012). This highlight that the results from this thesis (chapter 3 and 4), that demonstrate the large effect on biofilm development and production by shellfish reefs, may have consequences for costal function on a larger scale.

The positive interaction between the biofilm and shellfish reefs that I demonstrate in this thesis also indicates the potential for facilitative processes to create stabilising feedbacks in soft sediment systems (Eriksson et al. 2010). Blue mussel dominated reefs facilitate the biofilm locally and around the reef structures, and create

regions with high microphytobenthos biomass, nutrient uptake and primary production (Donadi et al. 2013a, Eriksson et al. 2017, chapter 3 and 4). At the same time, through resuspension and benthic pelagic coupling, the microphytobenthos is an important food-source for suspension feeders, such as the reef forming blue mussels and Pacific oysters themselves (de Jonge & Beusekom 1995, Ubertini et al. 2012). Thus, our results indicate that intertidal shellfish reefs create areas of high production and biodiversity that they themselves are favoured by, thus promoting self-reinforcing feedbacks. As ecosystem engineers they are therefore not only improving their own physical habitat, but are also gardening their own food source (Fig. 6.5). If the feedbacks are strong enough, it could lead to an alternative stable state dynamic where biological engineering enable reef dominated communities to exist under otherwise unfavourable external conditions (Scheffer et al. 2001; van de Koppel et al. 2001; Rietkerk et al. 2004; van de Koppel et al. 2005). Such facilitation feedbacks probably make soft sediment reef systems more resilient towards change, since the feedback may buffer the reef from natural stressors and fluctuating limitations (Eriksson et al. 2010). However, it may also make the reef ecosystem highly vulnerable for catastrophic regime shifts, caused by unpredictable and/or persistent disturbances that changes the reef so much that it disrupts the facilitation feedback, leading to accelerating collapse of the reef ecosystem (Folke et al. 2004). This indicates that the fundamentally best way to manage intertidal shellfish reefs is to make sure they do not go away by protecting them, because restoring them may be unexpectedly difficult. The presence of facilitation feedbacks means that for successful restoration of shellfish reefs, we may need to recreate ecological interactions; which is more difficult and complicated than just constructing physical structures.

Figure 6.5. The demonstrated network of positive interactions that may stabilise the shellfish reef-biofilm complex. Blue arrows show the facilitation feedbacks generated by physical biostabilisation of shellfish reefs. Shellfish reefs protect the sediment surface from hydrodynamic stress and decrease sediment erosion in their wake. This favours microphytobenthos and shellfish development (Donadi et al. 2013a, Donadi et al. 2013b, Donadi et al. 2014, Eriksson et al. 2017, chapter 3 and chapter 4). The orange arrow shows the facilitation feedback provided by chemical biostabilisation of the biofilm (e.g. Weerman et al. 2012). Biofilm increase the cohesiveness of the sediment by producing extracellular polymeric substances that glue the sediment grains together. This favours the development of the biofilm. The green arrow shows a trophic interaction. Resuspended biofilm is an important food source for shellfish in soft sediment ecosystems (de Jonge & Beusekom 1995, Ubertini et al. 2012).

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**#There are two ways of spreading light:
To be the candle or the mirror that reflects it**
-Edith Wharton-

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An investment in knowledge always pays the best interest.
-Benjamin Franklin-

SUMMARY

The ecology of soft-sediment systems is largely influenced by ecosystem engineering organisms. Ecosystem engineers modify their own environment and thereby extend and create habitats for other associated organisms. These associated organisms may have positive or negative effects on the engineering organisms themselves; creating a complex interaction network of positive and/or negative feedback loops that may stabilise and/or destabilise the configuration of the ecosystem. Thus, understanding the ecological interactions between ecosystem engineers and their associated species is required in order to manage and protect soft-sediment ecosystems. In this thesis, I studied the function of shellfish reefs for benthic algae on a tidal mudflat. I studied engineering effects both on the macroalgal meadows that inhabit the shellfish reefs, and on the benthic diatoms that form a biofilm on the surface of the mudflat.

I performed the studies of my thesis on the tidal flats of Schiermonnikoog by the means of bio-manipulation experiments creating small scale aggregations of reef- or bed-forming bivalves: the invasive Pacific oyster (*Crassostera gigas* or *Magallana gigas*), the blue mussels (*Mytilus edulis*), and the common cockle (*Cerastoderma edule*). The experiments were designed on a small spatial and short temporal scale to determine ecological processes. To support the experimental results, I also carried out field surveys where I identified multiple biological components and physical parameters: including the detailed species composition of seaweed and biofilm communities, chlorophyll *a* concentration and organic matter content of the sediment, and hydrodynamic conditions. In addition, I did a preliminary study based on the molecular identification of benthic diatoms, aiming at improving species identification and monitoring of benthic diatoms in the future.

In **Chapter 1**, I introduce my research about the ecological function of habitat structuring species on tidal flats. I introduce the biological aspects of tidal flats in the Wadden Sea and relate it to the very special and dynamic habitat that is created by soft sediment processes. The dynamic nature of soft sediments allows engineering organisms to shape their own habitat, which is in stark contrast to organisms inhabiting rocky shores. In particular, I focus on the interaction between intertidal shellfish reefs and algae (macro and micro-algae) communities in the Wadden Sea; as prominent examples of habitat forming ecosystem engineers and their fundamental effect on associated species in soft-sediment systems.

Chapter 2 develops the mechanistic understanding of the community structuring effects of the invasive Pacific oyster in the Wadden Sea, by demonstrating how it alter the energy flow and habitat- function of intertidal reef- communities. I discover that specific seaweed communities with fundamentally different functions developed on different types of shellfish reefs. On native blue mussel reefs, habitat forming and structurally complex fucoid canopies developed by means of entanglement in the byssus threads produced by the mussels; while on invasive Pacific oyster reefs, structurally simple and ephemeral green algae dominated communities developed instead. The chapter demonstrates the community structuring function and fundamental importance of blue mussel byssus threads for the intertidal ecology.

Chapter 3 unveil the importance of engineering effects of intertidal mussel beds for primary production, on large spatial and temporal scales. A combination of small-scale bio-manipulation experiments and field samplings across the tidal flat over several years, established that mussel beds transform the tidal flat into a power station for benthic primary producers; increasing production and standing biomass of algae both locally, and for 100s of meters in the wake of the reef.

In **Chapter 4**, I describe the effect of mussel reefs on the biodiversity and composition of benthic diatom communities. I discovered that bioengineering by the

mussel reef change the functional composition of the diatom community by promoting higher numbers of smaller sized cells and species. Small diatoms have higher growth and gross photosynthesis rates than larger, indicating that this explains the higher production and chlorophyll *a* concentration of the biofilm measured on the intertidal blue mussel reef in chapter 3. This finding highlights the potential for a positive feedback loop between bioengineering mussels and the biofilm; since suspended biofilm is an important food source for the reef forming mussels themselves.

Chapter 5 presents a preliminary study of molecular identification of benthic diatoms by implementing *rbcL* as the genetic marker. To support the sequencing results, I constructed a phylogenetic tree and the outcome showed high percentage of similarity. The recommendations for further study are to have sufficient amount of starting materials and that we need to have a general improvement of the reference database for benthic diatoms.

Last, in **Chapter 6**, I present the main findings of my thesis and discuss consequences for intertidal ecology:

- The effects of invasive species are not restricted to only direct effects of competition with organisms from the same ecological guild, but also result in cascading effects on organisms on other trophic levels.
- Ecosystem engineers fuel the primary production on the tidal flat
- Biogenic structures affect the composition of motile benthic diatom communities and change the function of the intertidal biofilm
- The *rbcL* gene is likely well qualified as a genetic marker for the molecular identification of benthic diatoms

To summarize, in this thesis I presented positive feedbacks of ecosystem engineers on biostability in soft sediment systems by demonstrating the large effect of shellfish reefs on biofilm development and production.

SAMENVATTING

De ecologie van los sedimentsystemen (slibafzettingen) wordt grotendeels beïnvloed door ecosysteemingenieurs (ook wel biobouwers genoemd). Ecosysteemingenieurs modificeren hun eigen leefomgeving en creëren zo habitats voor andere organismen in de levensgemeenschap, welke op hun beurt de biobouwers dan weer positief of negatief kunnen beïnvloeden. Hierdoor kan een complex interactienetwerk van positieve en/of negatieve terugkoppelingslussen ontstaan die de samenstelling van het ecosysteem kunnen stabiliseren en/of destabiliseren. Het begrijpen van de ecologische interacties tussen ecosysteemingenieurs en hun geassocieerde soorten is daarom vereist om slibecosystemen te beheren en te beschermen. In dit proefschrift heb ik de functie van schelpenbanken voor bentische (d.w.z. op de bodem levende) kiezelwieren (diatomeeën) op een wadplaat onderzocht. Ik heb de modifierende effecten onderzocht op zowel de zeewieren (macroalgen) die de schelpenbanken bewonen, als op de bentische diatomeeën die een biofilm vormen op het oppervlak van het wad.

Ik heb de studies van mijn proefschrift uitgevoerd op de droogvallende platen bij Schiermonnikoog door middel van biomanipulatie-experimenten waarbij kleinschalige aggregaties van rifvormende tweekleppigen (bivalven) werden gevormd: de invasieve (niet-inheemse) Japanse oester (*Crassostera gigas* of *Magallana gigas*), de (gewone) Mossel (*Mytilus edulis*) en de (gewone) Kokkel (*Cerastoderma edule*). De experimenten om ecologische processen te bepalen, zijn opgezet op een kleine ruimtelijke schaal en een korte tijdschaal. Om de experimentele resultaten te ondersteunen, heb ik ook veldstudies uitgevoerd waarbij ik meerdere biologische componenten en fysieke parameters identificeerde, waaronder de gedetailleerde soortensamenstelling van zeewier- en biofilmgemeenschappen, de chlorofyl a-concentratie en het organische stofgehalte van het sediment, en hydrodynamische

omstandigheden. Daarnaast heb ik een voorstudie gedaan op basis van de moleculaire identificatie van benthische diatomeeën, met als doel de soortidentificatie en monitoring van benthische diatomeeën in de toekomst te verbeteren.

In **Hoofdstuk 1** introduceer ik mijn onderzoek naar de ecologische functie van habitatstructurende soorten op getijdenplaten. Ik introduceer de biologische aspecten van wadplaten in de Waddenzee en relateer deze aan het zeer bijzondere en dynamische habitat dat ontstaat door processen in het losse sediment. De dynamische aard van losse sedimenten stelt ecosysteemingenieurs in staat om hun eigen habitat vorm te geven, wat in schril contrast staat met organismen die rotskusten bewonen. In het bijzonder richt ik me op de interactie tussen schelpenbanken en wierengemeenschappen (zowel macro- als micro-algen) in het getijdengebied van de Waddenzee. Dit zijn prominente voorbeelden van habitatvormende ecosysteemingenieurs en hun fundamentele effect op geassocieerde soorten in los-sedimentsystemen.

Hoofdstuk 2 ontwikkelt het mechanistische begrip van de gemeenschapsstructurende effecten van de invasieve Japanse oester in de Waddenzee, door aan te tonen hoe deze soort de energiestroom en de habitatfunctie van rifgemeenschappen in het intergetijdegebied verandert. Ik ontdekte dat specifieke zeewiergemeenschappen met fundamenteel verschillende functies zich ontwikkelden op verschillende soorten schelpenbanken. Op inheemse mosselbanken ontwikkelden zich habitatvormende en structureel complexe bedekkingen van fucoïde wieren (Blaaswier, Zeeëik) door middel van verstrikking in de door de mosselen geproduceerde byssusdraden. Op de uitheemse Japanse oesterbanken ontwikkelden zich daarentegen structureel eenvoudige en kortstondige door groene algen gedomineerde gemeenschappen. Het hoofdstuk demonstreert de gemeenschapsstructurende functie en het fundamentele belang van mosselbyssusdraden voor de intergetijdenecologie.

Hoofdstuk 3 onthult het belang van de habitatstructurende effecten van mosselbanken in het intergetijde voor de primaire productie, op grote ruimtelijke schaal

en grote tijdschaal. Een combinatie van kleinschalige biomanipulatie-experimenten en bemonsteringen op het wad gedurende meerdere jaren, toonde aan dat mosselbanken het wad transformeren tot een krachtcentrale voor benthische primaire producenten; de productie en de algenbiomassa nam toe, zowel lokaal als op honderden meters achter het rif (de mosselbank).

In **Hoofdstuk 4** beschrijf ik het effect van mosselbanken op de biodiversiteit en samenstelling van benthische kiezelwiergemeenschappen. Ik ontdekte dat habitatstructurering door de mosselbank de functionele samenstelling van de diatomeeëngemeenschap verandert door een toename van de aantallen kleinere cellen en soorten. Kleine diatomeeën hebben hogere groei- en bruto fotosynthese-snelheden dan grotere,. Dit verklaart de hogere productie en chlorofylconcentratie van de biofilm die gemeten werd op op de intergetijdemosselbank in Hoofdstuk 3. Deze bevinding benadrukt het potentieel voor een positieve terugkoppeling tussen bio-engineerende mosselen en de biofilm, want gesuspendeerde biofilm is een belangrijke voedselbron is voor de rifvormende mosselen zelf.

Hoofdstuk 5 presenteert een voorstudie van de moleculaire identificatie van benthische diatomeeën met behulp van het *rbcL*-gen als genetische merker. Om de DNA-sequentieresultaten te ondersteunen, heb ik een fylogenetische boom gereconstrueerd en de uitkomst vertoonde een hoog percentage overeenkomstigheid. De aanbevelingen voor verder onderzoek zijn om voldoende uitgangsmateriaal te hebben en tevens dat we een algemene verbetering van de referentie-DNA-databank voor bentische diatomeeën nodig hebben.

Tot slot presenteer ik in **Hoofdstuk 6** de belangrijkste bevindingen van mijn proefschrift en bespreek ik de gevolgen voor de intergetijde-ecologie:

- De effecten van invasieve soorten beperken zich niet alleen tot directe effecten van concurrentie met organismen van hetzelfde ecologische gilde, maar leiden ook tot trapsgewijze effecten op organismen op andere trofische niveaus.

- Ecosysteemingenieurs drijven de primaire productie op het wad op.
- Biogene structuren beïnvloeden de samenstelling van beweeglijke benthische diatomeeëngemeenschappen en veranderen de functie van de intergetijdebiofilm
- Het *rbcL*-gen is waarschijnlijk geschikt als een genetische merker voor de moleculaire identificatie van benthische diatomeeën.

Samenvattend presenteerde ik in dit proefschrift positieve terugkoppelingen van ecosysteemingenieurs op de biostabiliteit in los-sedimentsystemen door het grote effect van schelpenbanken op de ontwikkeling en productie van biofilms aan te tonen.

RINGKASAN

(Bahasa Indonesia)

Ekologi sistem sedimen lunak sebagian besar dipengaruhi oleh organisme rekayasa ekosistem (bioengineering), dimana organisme ini memodifikasi lingkungan mereka sendiri yang pada akhirnya memperluas dan menciptakan habitat bagi organisme-organisme terkait lainnya. Organisme yang terkait ini mungkin memiliki efek positif atau negatif pada organisme rekayasa (bioengineering) itu sendiri: menciptakan jaringan interaksi yang kompleks dari lingkaran umpan balik positif dan/atau negatif yang dapat menstabilkan dan/atau mengacaukan konfigurasi ekosistem. Dengan demikian, pemahaman tentang interaksi ekologis antara organisme rekayasa ekosistem (bioengineering) dan spesies terkait lainnya diperlukan untuk mengelola dan melindungi ekosistem sedimen lunak ini. Dalam disertasi ini, saya mempelajari fungsi terumbu kerang untuk alga bentik di area substrat berlumpur di zon pasang surut. Saya mempelajari efek dari bioengineering terhadap komunitas rumput laut (makroalga) yang menghuni terumbu kerang, dan pada diatom bentik yang membentuk biofilm di area permukaan di substrat berlumpur.

Saya melakukan studi doktoral ini di zon pasang surut *Schiermonnikoog* melalui eksperimen bio-manipulasi yang menciptakan agregasi skala kecil dari bivalvia pembentuk terumbu atau paparan : tiram Pasifik invasif (*Crassostrea gigas* atau *Magallana gigas*), kerang biru (*Mytilus edulis*), dan kerang biasa (*Cerastoderma edule*). Eksperimen dirancang pada skala kecil spasial dan temporal pendek untuk menentukan proses ekologi. Untuk mendukung hasil eksperimen, saya juga melakukan survei lapangan di mana saya mengidentifikasi beberapa komponen biologis dan parameter fisik: termasuk komposisi spesies rumput laut dan komunitas biofilm secara detail, konsentrasi klorofil *a* dan kandungan bahan organik sedimen, dan kondisi

hidrodinamika. Selain itu, saya melakukan studi pendahuluan berdasarkan identifikasi molekuler diatom bentik, yang bertujuan untuk meningkatkan identifikasi spesies dan pemantauan diatom bentik di masa depan.

Dalam **Bab 1**, saya memperkenalkan penelitian saya tentang fungsi ekologis spesies pembentuk habitat di zon pasang surut. Saya mengungkapkan aspek biologis di zon pasang surut – *Wadden Sea* dan menghubungkannya dengan habitat yang unik dan dinamis yang dibentuk oleh proses sedimen/substrat lunak. Sifat dinamis dari sedimen/substrat lunak ini memungkinkan organisme rekayasa (bioengineering) untuk membentuk habitat mereka sendiri, yang sangat kontras dengan organisme yang menghuni pantai berbatu. Secara khusus, saya fokus pada interaksi antara terumbu kerang intertidal dan komunitas alga (makro dan mikroalga) di *Wadden Sea*; contoh dominan dari dalam ekosistem pembentuk habitat dan mempunyai efek mendasar pada organisme sistem sedimen/substrat lunak.

Bab 2 mengembangkan pemahaman mekanistik tentang efek penataan komunitas dari invasif tiram Pasifik di *Wadden Sea* dengan menunjukkan bagaimana organisme tersebut mengubah aliran energi dan fungsi habitat komunitas terumbu di zon intertidal. Saya menemukan bahwa komunitas rumput laut tertentu dengan fungsi yang berbeda secara fundamental berkembang di berbagai jenis terumbu kerang. Di terumbu orisinal kerang biru, pembentukan habitat dan kanopi furoid (alga cokelat) yang kompleks secara struktural dikembangkan dengan cara terjatuh dalam benang byssus yang dihasilkan oleh kerang biru; sementara di terumbu tiram Pasifik yang invasif, komunitas yang didominasi alga hijau (*Ulva* sp.), sebagai gantinya. Bab ini menunjukkan fungsi penataan komunitas dan kepentingan mendasar dari benang byssus komunitas kerang biru untuk ekologi intertidal.

Bab 3 mengungkap pentingnya efek rekayasa pada skala spasial dan temporal yang besar dari paparan/terumbu kerang biru yang terletak di zon intertidal untuk produktivitas primer. Kombinasi dari percobaan bio-manipulasi skala kecil dan

pengambilan sampel di lapangan, di daerah pasang surut selama beberapa tahun, menetapkan bahwa paparan/terumbu kerang biru mengubah daerah pasang surut menjadi daerah penghasil energi bagi produsen primer bentik; meningkatkan produksi dan biomassa tegakan alga secara lokalitas, dan dalam jangkauan 100-an meter di belakang paparan/terumbu.

Di **Bab 4**, saya menjelaskan pengaruh terumbu kerang terhadap keanekaragaman hayati dan komposisi komunitas diatom bentik. Saya menemukan bahwasanya bioengineering oleh terumbu kerang biru mengubah komposisi fungsional komunitas diatom dengan cara menaikkan jumlah sel dari spesies yang berukuran kecil dalam jumlah yang lebih banyak. Diatom berukuran kecil ini memiliki pertumbuhan dan laju fotosintesis bruto yang lebih tinggi; hal ini dijelaskan dalam Bab 3 yang menunjukkan bahwa produksi dan konsentrasi klorofil yang lebih tinggi dari biofilm terukur di kerang biru intertidal. Temuan ini menilik potensi loop umpan balik positif antara efek bioengineering kerang biru dan biofilm; yang mana biofilm yang tersuspensi dalam substrat merupakan sumber makanan penting bagi kerang biru pembentuk terumbu itu sendiri.

Bab 5 mengemukakan studi pendahuluan identifikasi molekuler diatom bentik dengan menerapkan *rbcL* sebagai penanda genetik. Untuk mendukung hasil sekuensing, saya membuat pohon filogenetik dan hasilnya menunjukkan persentase kemiripan yang tinggi. Rekomendasi untuk studi lanjutan adalah memiliki jumlah (kuantitas dan kualitas) sampel yang cukup dan perbaikan umum dari database referensi untuk diatom bentik.

Terakhir, di **Bab 6**, saya menyajikan temuan utama dari thesis doctoral ini dan mendiskusikan dampaknya bagi ekologi intertidal :

- Efek spesies invasive tidak hanya terbatas pada efek langsung dari kompetisi dengan sesama spesies di habitat yang sama, tetapi dipengaruhi juga oleh efek cascading pada organisme di tingkat trofik lainnya.
- Organisme pemodifikasi habitat mendorong produktifitas primer di daerah/dataran pasang surut.
- Struktur biogenik terumbu moluska mempengaruhi komposisi komunitas diatom benthik, yang mempunyai kemampuan untuk berpindah tempat (motil) dan mengubah fungsi biofilm di intertidal zon.
- Gen *rbcL* berpeluang besar sebagai penanda genetic yang berkompeten dalam studi identifikasi molekular diatom benthik.

Sebagai ringkasan, dalam thesis doctoral ini saya menyampaikan respon positif dari organisme yang berkemampuan untuk memodifikasi biostabilitas di ekosistem intertidal dengan substrat berlumpur, dengan menunjukkan pengaruh terumbu moluska pada perkembangan dan produksi biofilm dari alga benthik.

#Sunrise at Schiermonnikoog (©R. Andriana)

#Hakuna Matata!

- - no worries - -

-Jambo- The Lion King' song-

ACKNOWLEDGEMENT

Now, last but not least, I would like to thank everyone who inspired, motivated and contributed to the impressive experience of my PhD. Every single step was a learning process for me: learning about the culture, surviving from the rollercoaster's Netherlands weather, allowing myself to 'fall down' and 'get up' again not only with my two feet but also with encouragement, support and help from people around me.

Firstly, I am profoundly thankful to my supervisors **Klemens**, **Joana** and **Birte** for being very patient, guiding me through this PhD journey.

First time, I came to the Netherlands was in June 2014, and had a chance to meet up **Klemens** in person, at his office in Groningen. A short conversation about the opportunity to join his lab as PhD candidate, and you were agreed to be my promoter/supervisor if I succeeded to get scholarship for my study. This was the beginning of this 6-year PhD journey (2015-2021) that I will never forget. During this journey, once you reminded me, 'Do not force yourself too hard, Rosyta. Do things slowly, one at the time'; that was your advice when I started to make a 'to-do list' due to some datelines. Now, those words become my self-reminder. I admire your critical thinking, your teaching and supervising styles; you let me lead my own way and explore my hidden potential. So yes, the production of this thesis is a proof of your excellent leadership! Thank you also for taking me on some trips to Fiskebäckskil, Sweden, I had so much fun and certainly learned and explored new things!

In my third year of PhD, I had the opportunity to work together with **Joana**, by joining Molecular Methods in Ecology and Evolution (MMEE) Course, learning how to apply molecular approach in ecological studies. We run a preliminary study of molecular identification of benthic diatom and I am happy and proud that for a small project, it turned out to be a chapter in this thesis. Thank you for all your guidance and feedback!

Birte, thank you for the collaboration, correspondence and your ideas and comments in improving the manuscripts. You gave me a warm feeling and support in every contact that we had. Thank you for this!

Secondly, I thank the Indonesia Endowment Fund for Education (**LPDP**), the Ministry of Finance Republic of Indonesia for PhD financial support. *Terima kasih banyak* Ibu Ratna Prabandari and the staffs at the Ministry of Finance Republic of Indonesia who have ensured that my study goes well without any significant obstructions.

I want to thank my co-authors : **Birte, Klemens, Friederike, João, Javier, Serena, Maria, Isabelle, Stella, Joana** for your contributions to the manuscripts: comments, feedback and also assisting in the field and laboratory.

Isabelle and **Casey**! I'm so blessed and proud to have you, lovely people, in my journey. We have so much fun in our little office, fieldworks and even during our hanged-out time – which not so often but quality that matters. Our differences form a balance; filling each other's holes that how I feel– love you two so much! **Isabelle**, I admire your mindset, choices and how you inspire others without you noticing it. '*Just keep running, Rosy.. don't stop!*'- that was what you told me when I had cold feet during my marathon preparation; which I keep in my mind in every competition that I participated in. You have a gift to make people feel secure, comfortable and loved. Thank you for all of your kind and lovely messages – light up my day, for sure! And **Casey**, you are ROCK! You are the *coolest -lovely* person that I ever met. Yes, what a unique combination; you are a strong woman and yet you have warm and caring persona. We share a lot of things in that little office, personal stuff, serious matters to hilarious things, and of course not forgetting the gossips. Oh ya, the drinks; you always offer to prepare the drinks during occasions – Yes, our private bartenders! I love it! Thank you for being in my circle!

Friederike and **João**, you were my first companions on my first year of PhD journey. My first buddy on my first fieldwork onto the mudflat at Schiermonnikoog was you, **Friederike**; thank you for being patient and helping me out at the mudflat when I got stuck (a lot of times), and letting me know what to watch out during the walk on the

mudflat. I have learned so much from you, during fieldwork, laboratory sessions and of course your work ethic— about being a good researcher. And **João**, thank you for helping me to collect samples at Schiermonnikoog despite the nasty weather, I appreciated it so much. Thank you so much for guiding me on the first 2-years of my PhD, I am blessed to get the chance to know you both.

Hinke, thank you so much for your help, time and attention during my study in Groningen. Our chats were so diverse, from yoga, foods, weather, sports until values of life - I love it! **Stella**, thank you for sharing your knowledge about the equipments and cultures. You also introduced me to the beauty of Schiermonnikoog, the dunes, plants and beautiful hidden places on the mainland. **Corine**, I want to thank you for your help with all subjects concerning my PhD.

And, we call it the *third floor* group: **Xenia, Vania, Andrea, Yacine, Wensi, Nürja, Marlene, Lucia, Dani, Jorn, Fiona, Sorsha, Simona, Anna, Henrique, Jorge**, and everybody else of **the third floor** of Linnaeusborg for the support and great times that we had; lunch, coffee breaks, hanging out by Kroewa Thai, boating around Groningen, and partying at Casey's. Special thanks to **Xenia, Vania, Nürja, Marlene** for helping out to collect samples on the mudflat, when Vania invented that running was the most efficient way of avoiding sinking on the mudflat; at the end, we had a great time there! Also **Jorge** and **Yacine** for coaching me with R-program and phylogenetic matters, and Yacine, thanks again for keeping my spirit up at the last moment of completing this thesis.

Mas **Adjie, Edisa, Stefanie** from Microbial Community Ecology group, I want to thank you all for your assistance on my small project at your laboratory. You have been very helpful in providing all the information and materials that I needed, even a personal help in running the experiment. Thank you so much for this!

Jillian, Affendi, Serina and **Shalan**, my Cikgu's and best friends - **Jill**, you took me for the first time to Port Dickson and taught me how to do quadrat and transect lines'

samplings; ‘Boss’ **Fendi**, as my *private* dive instructor who once said ‘*Yes, when you dive you always have a buddy but remember.. the one who can save you, is yourself alone*’; Kak **Ina**, who reminded me – *just be yourself, Ochi.. no need to change just to satisfy others, because you are beautiful just the way you are*; Oom **Lan**, always care about others, make jokes and calm us down when we were at the tip of iceberg - you all played a big role to what I have achieved today. We always have so much fun when we were together, especially during fieldwork – diving, processing samples with UB’40 music as a background, and of course, lots of delicious Malay foods. Thank you for everything and I love you All!

Stefano, thank you for your continuous support! From the first time I knew you until now, I can say that you always be there for me, and thank you for translating the summary and abstract into Dutch on such short notice. Nogmaals, heel erg bedankt, lieve Stef!

Finally, all of this hard work will not come true without the tremendous support and love of my beloved family in Indonesia and the Netherlands, and, Yes, I have been blessed for having them in my life.

Mama & (almarhum) Papa : (untuk Papa, yang sudah mendahului kita semua, *rest in peace*) terima kasih untuk kasih sayang dan support yang selama ini sudah diberikan. Semua pelajaran hidup yang Mama dan Papa ajarkan, telah membentuk sosok pribadi Ochi dengan pencapaian yang sekarang ini. Doa restu dan wejangan Mama menjadi semangat Ochi untuk menyelesaikan sekolah ini; beruntung dengan kemajuan teknologi sekarang ini, kita bisa video-call, kirim pesan gratis, sekaligus berbagi resep masakan, atau sekedar bertanya ‘*masak apa hari ini, nduk*’. Hanya doa dan ucapan terima kasih yang bisa Ochi panjatkan untuk Mama: *matur suwun nggeh, sungkem kagem Mama*, panjang umur dan sehat-sehat slalu ya Ma. Ochi sayang Mama, Papa, *berkah dalem*.

To *sayangku*, lovely men in my life: **Rinus & Dennis**, my husband and son. Thank you for supporting me and filling my days with your unconditional love, for believing in me,

and also for being patient throughout this 6-year process! During my drowning time with my laptop, Rinus, you never forget to ask if I need anything, offering a cup of tea or snacks, and Dennis, coming to the room, occasionally, just only for hugging and kissing me, thank you both for your warm affection. I praise the Lord for having you two in my life.

Vanaf de eerste keer dat ik Nederland bezocht kreeg ik van jullie allemaal een warm en liefdevolle ontvangst: **Elly, Frans, Thea en Jan**. Jullie gaven mij een thuisgevoel en accepteerden mij, zoals ik ben. Jullie betrokken mij in jullie leven, alsof wij elkaar al jaren kenden. Jullie steunden mij, niet alleen met mijn studie, maar ook met koken, sport en andere dingen. Jullie stuurden mij altijd een ‘veel succesbericht’ voor elke wedstrijd en altijd gretig om de gerechten van de Indonesische keuken te proeven. Ik wil jullie allen danken voor jullie liefde en steun!

Mas Iwan, Mbak Minda, Dek Topan, Dek Riri, Khayla, Airin, Vina: Mas, Mbak, Adek-adekku, keponakanku sayang, di Jakarta dan Surabaya, terima kasih untuk doa dan semangat yang sudah dikirimkan dari jauh. For **Indi, Ed and Angel**, thank you for your supports in times of happy and sorrow.

Fred, Marga, Luuk, and Amber, firstly, I am grateful that our friendship has grown further, not only as a good friend but now as a family. It started when I landed in the Netherlands for the second time, in 2015, you welcomed us, me and Dennis, with your warm hearts and helped me take care of Dennis, when I was away for fieldwork. The relationship continued with birthday celebrations and Christmas dinners, and now the dinner is a family tradition that we celebrate every year. Thank you for your love and genuine support!

Finally, I want to thank everyone for their huge contributions in finalizing this thesis and also for those who have interest in reading it.

Thank you – Dank jullie wel – Matur nuwun - Terima kasih banyak.

The dunes in the National Park Schiermonnikoog (©R. Andriana)

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Rosyta Andriana obtained her bachelor's degree in Marine Science Technology in 2001 from the Faculty of Fisheries and Marine Science, Bogor Agricultural University (IPB), Indonesia. In completing her bachelor's degree, she conducted a small-scale observation of Gastropod distribution at the river mouth of Cimandiri river, Pelabuhan Ratu, Sukabumi, West Java, and produced an essay.

After 4 years, she moved to Kuala Lumpur, Malaysia to continue her master degree (by reseach) at Institute of Biological Sciences and worked as research assitant at Algae Research Laboratory (Prof. Dr Phang Sieuw Moi) and Institut of Ocean and Earcth Science (IOES) of University Malaya (UM) (2005-2011). Here, she performed field and laboratory experiments of seaweed and seagrass ecology; for her master degree, she designed and carried out an ecological observation of spatial and temporal distribution of *Sargassum* sp., brown algae, at Port Dickson, Negeri Sembilan, Peninsular Malaysia, and in her free time, she was actively involved with the local- non profit organization, Kelab Alami (Tanjung Kupang, Johor, Malaysia) in educating coastal youth community.

Returned to Indonesia in 2011, she taught Biology in the Indonesian national curriculum and Cambridge's at Bilingual Junior-Senior Highschools in Bekasi and West Jakarta (2011-2015).

In December 2014, she received scholarship from the Indonesia Endowment Fund for Education (LPDP) to carry out her PhD program at Groningen University (RUG). Within 6 months, in June 2015, she started her PhD program at Prof. Dr. Britas Klemens Eriksson's Marine Ecology Laboratory by implementing ecological experiments, both in the laboratory and field of algae community; in the Netherlands, her 'playground' was the intertidal flat.

Yin & yang.