Diversity and Ecosystem Functioning in Estuarine Intertidal Microphytobenthos

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Diversity and Ecosystem Functioning in Estuarine Intertidal Microphytobenthos

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General Introduction
Chapter 1

Biodiversity and Ecosystem Function

Humanity has become a major biogeochemical force and human domination of the planet has caused massive destruction and fragmentation of natural habitats, eutrophication, climate change, acidification, etc (Vitousek et al., 1997, Tilman et al., 2001b). Human activities have resulted in dramatic biodiversity loss and have altered ecosystems on a global scale (Chapin et al., 2000, Sala et al., 2000). The estimates and predictions of this ongoing species loss have raised concern about the consequences of biodiversity loss on ecosystem properties and the goods and services these ecosystems provide to humanity (Diaz et al., 2006, Worm et al., 2006).

Biodiversity

Biodiversity is supposed to affect ecosystem functioning because the richness and identity of the species present in an ecosystem determine the functional traits available in an ecosystem. These functional traits may govern flows of energy and matter and may influence the response of ecosystems to abiotic conditions such as disturbance and nutrient availability. The constituents of biodiversity that determine the presence and importance of functional traits in an ecosystem include species richness, their relative abundance and identity, species interactions and the temporal and spatial variation in these properties (Symstad et al., 2003).

In order to understand the effects of species loss on ecosystem functioning, we first need a solid knowledge of the actual biodiversity. Assessing diversity is not as trivial as it may seem at first, especially in microbial communities. Traditionally, species have been defined on the basis of discontinuities in morphological and ultrastructural properties and can therefore be designated as “morphospecies”. A multitude of molecular studies however have demonstrated that traditionally defined morphospecies often consist of several genetically distinct groups (Darling et al., 2004, Hebert et al., 2004, Slapeta et al., 2006). Such genetically distinct but morphologically indistinguishable species are called “cryptic
species”, whereas the term “pseudocryptic species” refers to the occurrence of morphologically similar species which at a closer look do exhibit subtle morphological differences (Mann & Evans, 2007).

(Pseudo)cryptic species appear to be widespread, especially among microalgae. Studies comparing reproductive, molecular, physiological or ecological characters of several strains belonging to the same microalgal morphospecies almost invariably demonstrated the occurrence of (pseudo)cryptic species (Knowlton, 1993, Mann, 1999, Knowlton, 2000, Saez et al., 2003, Beszteri et al., 2005, Slapeta et al., 2006, Amato et al., 2007, Vanormelingen et al., 2008). As a consequence, our current estimates of microbial diversity almost certainly constitute a serious underestimation of actual biodiversity (Mann, 1999).

As mentioned above, biodiversity determines the functional traits that are present in a community. In most cases however, it is unknown whether (pseudo)cryptic species differ in their ecological preferences or whether they are functionally equivalent. This notion is not only important for questions relating biodiversity and ecosystem function (BEF), but also to understand mechanisms that promote cryptic species coexistence. Niche differentiation is clearly important for species coexistence, but in some cases there is a marked similarity in coexisting species. Scheffer & van Nes (2006) used a classical Lotka-Volterra model to demonstrate that competing species can evolve to being more similar, instead of less. According to this model coexisting species are either sufficiently similar or sufficiently different.

Only a few studies have verified ecological differentiation between (pseudo)cryptic protist species and suggest possible differentiation in light requirements (Rodriguez et al., 2005), temperature preference (Boenigk et al., 2007) and susceptibility to parasites (Mann, 1999).

Development of the Biodiversity – Ecosystem Functioning Field

Ecology historically treated biodiversity as a corollary of the abiotic environment and ecosystem functioning (Gross & Cardinale, 2007). One of the most important ecosystem functions that is expected to regulate biodiversity is productivity. Productivity is used as a proxy for limiting
resource supply rate which regulates the number of competing species that can locally coexist (see species-energy theory and resource ratio theory Tilman, 1977; Wright, 1983) During the last two decades this unidirectional view has been altered and diversity is no longer considered as just a result of environmental influences. On the contrary, it has been shown that diversity can regulate habitat formation (Jones et al., 1994, Wright & Jones, 2006), elemental fluxes and productivity (Naeem, 2002, Hooper et al., 2005).

This paradigm shift was elicited by the current biodiversity crisis, but the idea that diversity can influence ecosystem functioning it is not a fully novel view. The first experiment linking diversity to productivity dates back to the early 19th century. This experiment was performed in 1816 by George Sinclair, the head gardener to the Duke of Bedford, in the country gardens of Woburn Abbey (Bedfordshire, UK). The design comprised 242 plots which were sown with different numbers of plant species. The aim was to determine the role of diversity and under which conditions diversity matters (e.g. different soil types). This experiment was later described by Charles Darwin in his work “The Origin of Species” and lead him to conclude “it has been experimentally proven that, if a plot of ground be sown with one species of grass, and a similar plot be sown with several distinct genera of grasses, greater number of plants and greater weight of dry herbage can thus be raised” (experiment described in Hector Hooper, 2002).

Later on, Elton (1958) and MacArthur (1955) initiated the debate about the relation between diversity and stability of ecosystems, stating that more diverse communities are more resilient and stable. The current focus on biodiversity and ecosystem functioning was instigated by Ehrlich and Ehrlich (1981) who introduced the “Rivet hypothesis” which compared the diversity of an ecosystem with the rivets (clinch-nails) of an airplane. Not devoid of any sense of drama, the authors suggest that some redundant rivets could pop out without the wings being compromised, but when rivet loss rises, catastrophe is near. In parallel, a few extinctions in natural communities may not cause any shift in functioning, but the loss of too many species could result in declining ecosystem functioning. Several other forms of BEF relations have been proposed (Naeem, 1998, Schlapfer et al., 1999) such as the proportional (linear) loss hypothesis which states that ecosystem functions decline linearly with species loss (fig. 1).
Introduction

The redundancy hypothesis (Walker, 1992) assumes that ecosystem process rates increase with increasing diversity, but only to a certain level at which new species are redundant and do not supply additional contributions to ecosystem processes. According to this hypothesis, initial species loss has no effect, but at a certain level of species loss functioning diminishes. The keystone species hypothesis is based on the observation that certain species make a unique and often extreme contribution to ecosystem function. Another hypothesis predicts that the change in ecosystem function is context dependent and therefore idiosyncratic (the idiosyncratic hypothesis, Lawton, 1994). Such pattern could occur when species make different contributions depending on e.g. disturbance level, nutrient availability, etc.

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Early hypotheses

Figure 1. Different shapes describing the relation between biodiversity and ecosystem functioning (Naeem et al., 2002)
Modern BEF experiments

More recent experimental testing of BEF relations started in the 1990s (Naeem et al., 1994, Tilman et al., 1996, Hector et al., 1999). These BEF studies manipulated biodiversity under controlled conditions and evaluated the effect of diversity on ecosystem functioning, mainly primary productivity. Some of the early BEF experiments (Naeem et al., 1994, Tilman & Downing, 1994) such as the ECOTRON experiment (Naeem et al., 1994) were criticized by Huston (1997) and Aarssen (1997) because these experiments manipulated diversity indirectly and changes in richness were confounded with changes in the species composition. Later on, the experimental design of most BEF experiments was optimized so that the total initial biomass of producers was held constant across several diversity levels. Furthermore, combinations of species should be selected at random and both species richness and species composition should be replicated (replicated combinatorial design, Giller et al., 2004).

Since the initial BEF experiments, almost 200 papers describing biodiversity ecosystem function experiments have been published (Cardinale et al., 2011). This wealth of data has been extensively reviewed (Loreau et al., 2001, Hooper et al., 2005, Srivastava & Vellend, 2005, Reiss et al., 2009, Loreau, 2010) and several meta-analyses have been performed (Balvanera et al., 2006, Cardinale et al., 2006, Worm et al., 2006, Cardinale et al., 2007, Cadotte et al., 2008). The compiled evidence provides persuasive support for a positive relation between diversity and the efficiency by which (mainly) plants and algae capture limiting resources and convert these into biomass (Cardinale et al., 2011). These experiments also provided support for the diversity-stability hypothesis which states that more diverse communities can better resist disturbance (Tilman & Downing, 1994, McCann, 2000). Stability incorporates both the resilience (speed of recovery) and the actual resistance. The idea behind this hypothesis is that more diverse communities harbor more functional traits and that during an environmental perturbation some species with specific traits will compensate for the reduced performance of other species.
Mechanisms Involved

After establishing the positive biodiversity - ecosystem function relation, one of the next goals was (and still is) to elucidate the mechanisms responsible for the positive biodiversity effects. Ecological theory suggests that positive BEF relations are caused by three mechanisms: sampling or selection probability effects, niche complementarity and facilitation (Loreau et al., 2001). The sampling effect refers to the increased probability of the presence of species with particular, functionally important traits with increasing species richness (Huston, 1997). Niche complementarity occurs when species have different resource requirements, resulting in lower competition from interspecific neighbours than from conspecifics. This may lead to a more complete resource use by more speciose communities (Fridley, 2001). Facilitation occurs when a species modifies the environment in a way favourable for other co-occurring species (Vandermeer, 1989). Niche complementarity and facilitation are collectively referred to as ‘complementarity’, as it is often unclear which mechanism prevails.

Although many researchers claim that niche partitioning or facilitation mechanisms are responsible for positive BEF relations, there is rarely conclusive proof. Cardinale et al. (2011) recently performed a meta-analysis on BEF experiments covering 192 peer-reviewed papers and found that not even half of the claims for niche complementarity are backed by any direct statistical test or direct experimental verification.

With the available data, they conclude that complementarity appears to play the primary role in driving positive diversity effects in aquatic ecosystems, while in terrestrial ecosystems both complementarity and sampling effects were present.

The occurrence of complementarity is generally inferred indirectly by comparing mixture yields with the expected yield based on the monocultures of the component species (Loreau & Hector, 2001). However, at present, we know very little about the biological mechanisms that are responsible for niche complementarity and facilitation. Therefore we need more direct tests of mechanisms rather than merely calculating the difference between expected and observed yield.

A better understanding of diversity effects will also be gained by getting better insights in species interactions, both with respect to the identity of these interactions and the interaction strengths. Species interactions (e.g. facilitation, mutualism, competition, and predation) have the potential to
alter biodiversity effects. Recently, a modelling approach (Kirwan et al., 2009) has been developed to test among different hypotheses on how species interactions can modify diversity effects. By observing patterns in species interactions, these models suggest which species mechanisms may occur, but they do not produce evidence for specific mechanisms. The only way to estimate the importance of a specific species interaction is to experimentally manipulate the factors that influence interactions such as facilitation and competition. Mechanistic evidence for facilitation has been demonstrated for N-fixing legumes (Temperton et al., 2007) and larvae of suspension-feeding Trichoptera (Cardinale et al., 2002), but further direct evidence for facilitation that enhances positive diversity effects is lacking (Bulleri, 2009).

Biodiversity - Ecosystem Functioning in Estuarine Intertidal Microphytobenthos

Until recently most BEF experiments were focused on grassland communities, e.g. the Minnesota Cedar Creek biodiversity experiments (Tilman et al., 2001a), the BIODEPTH experiment (Hector et al., 1999, Spehn et al., 2005) and the Jena biodiversity experiment (Roscher et al., 2004). However, during the last few years researchers have begun to use microbial systems for BEF experiments. These have a few clear advantages for BEF research, such as easy control and replication, the possibility to run experiments over many generations in a short time period (Bell et al., 2005, Replansky & Bell, 2009, Striebel et al., 2009, Gravel et al., 2011).

In this thesis I focus on the biodiversity and ecosystem functioning of estuarine intertidal microphytobenthic (benthic microalgae) communities. Estuarine and coastal ecosystems provide some essential ecosystem services such as carbon fixation, food production, disturbance regulation and coastal protection and nutrient cycling (Costanza et al., 1997). Despite the fact that these habitats cover relatively small areas on a global scale, their importance for several biogeochemical processes is disproportionately large (Costanza et al., 1997). In many marine and estuarine ecosystems intertidal and subtidal
Introduction

Sediments support the growth of dense biofilms of benthic microalgae, which can provide up to 50% of the total primary production in estuaries (Underwood & Kromkamp, 1999). Microphytobenthos has many essential functions for these ecosystems: they are the basis of the food chain for many coastal species (MacIntyre et al., 1996), mediate nutrient fluxes between sediments and the water column (Sundbäck et al., 2000) and stabilize sediments and as such enhance the resistance of sediment towards erosion (de Brouwer et al., 2000, Yallop et al., 2000).

We focused on benthic diatoms as they are often the dominant constituent of intertidal mudflats in temperate regions (Admiraal, 1984). Many descriptive field studies have assessed the diversity of these benthic diatom communities and tried to explain species distribution in terms of a set of abiotic environmental parameters. Other studies on the functioning of these biofilms focused mainly on their impact on biogeochemical cycles (e.g. Herman et al., 2000, Middelburg et al., 2000, Risgaard-Petersen, 2003), sediment stabilization (de Brouwer et al., 2000, Yallop et al., 2000) and photophysiology of the microalgae (e.g. Serodio et al., 1997, Barranguet et al., 1998, Kromkamp et al., 1998, Perkins et al., 2001). Very few studies however combined functional studies with information on species identity and richness (but see Nilsson & Sundbäck, 1996, Underwood & Provot, 2000, Underwood et al., 2005, Jesus et al., 2009). Therefore we have a limited ability to link diatom biofilm functioning with the diversity of these diatom communities (Underwood, 2005b).

A recent field study by Forster et al. (2006) on intertidal mudflats in the Westerschelde estuary (The Netherlands) showed a site specific relation between primary productivity and diversity, with either a positive or a unimodal relation between both. Yet, this field study is correlative and has no experimental control on confounding factors which restricts conclusions about causative relations between diversity and ecosystem functioning. Moreover, such field studies can not give further information on the mechanisms responsible for diversity-mediated ecosystem functioning.
Chapter 1

Aims and Outline

The general objective of this thesis was to obtain a better understanding of the diversity and ecosystem functioning, and the relationship between both, in estuarine benthic diatom communities. More specifically, we wanted to find out (1) whether cryptic diversity occurred in dominant benthic diatom species (*Navicula phylepta*, *Cylindrotheca closterium* and *C. fusiformis*) and how (pseudo)cryptic variation is correlated with ecological differentiation; (2) whether the ecological niches of these cryptic species (i.e. *Cylindrotheca* spp.) are more conserved among more related lineages than between more distantly related lineages; (3) how taxonomic and functional diversity is regulated by hydrodynamic disturbance. (4) to what degree and how (positive or negative) diversity affects the functioning of benthic diatom communities with respect to their productivity (overall productivity and growth of individual taxa); (5) what mechanisms are involved in the observed BEF relations.

In Chapter 2: We investigated genetic, morphological and ecophysiological variation in estuarine populations of the widespread benthic diatom *Navicula phylepta*. Genetic variation was assessed by using sequences of the *rbcL*, ITS rDNA and 18S rDNA. We assessed ecophysiological differences by determining salinity tolerances of the different strains.

In Chapter 3 we focused on global strains of the diatom genus *Cylindrotheca*. We composed a set of strains from a wide range of coastal habitats, from coastal plankton to sea ice and intertidal mudflats. We determined the phylogenetic history of 40 *Cylindrotheca* strains and quantified their fundamental temperature niches using laboratory experiments. We then assessed if closely related strains tend to be more similar to each other than to more distantly related ones and therefore if a phylogenetic signal can be found in climatic niches.

Chapter 4: describes the relations between the hydrodynamic disturbance, species diversity and functional group turnover in estuarine intertidal microphytobenthos. We composed an extensive data set of benthic diatom assemblages from the Scheldt estuary covering a wide range of hydrodynamic disturbance. We tested whether diversity -
disturbance patterns in estuarine microphytobenthos are consistent with the Intermediate Disturbance Hypothesis.

In Chapter 5 we experimentally explored the importance of species diversity for the productivity of intertidal benthic diatoms. We used naturally co-occurring diatom species to experimentally assemble communities with decreasing diversity. We measured biodiversity effects by calculating overyielding (occurring when a mixture of species performs better than its monocultures). We explored the potential mechanisms for biodiversity effects using the additive partitioning equation of Loreau & Hector (2001) and by conducting an additional culture experiment to elucidate potential facilitative interactions between different species.

Chapter 6: We demonstrate that cultures of the benthic marine diatom *Nitzschia* cf. *pellucida* exude metabolites with strong allelopathic effects. Competing diatom species were inhibited in their growth and photosynthetic efficiency. In addition, we show reciprocal, density dependent allelopathic interactions between *N. pellucida* and two other marine benthic diatom species, *Entomoneis paludosa* and *Stauromella* sp.

Chapter 7: By chemically characterizing the gas-phase of cultures of the marine benthic diatom *Nitzschia* cf. *pellucida* using headspace solid phase microextraction (HS-SPME) coupled to GC-MS, we show that this diatom exudes a diverse mixture of volatile iodinated and brominated metabolites including the new natural product cyanogen bromide (BrCN) which exhibits pronounced allelopathic activity. We show that allelopathic effects are H_2O_2 dependent and link BrCN production to haloperoxidase activity.

Chapter 8: General discussion and perspectives
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Introduction


Chapter 1


Chapter 2

Ecological Differentiation between Sympatric Pseudocryptic Species in the Estuarine Benthic Diatom *Navicula phyllepta* (Bacillariophyceae)

Chapter 2

Abstract
The occurrence of cryptic and pseudocryptic species, often living in sympatry, is widespread among microalgae. This phenomenon raises important questions about niche partitioning between these closely related species. To date, however, few studies have addressed the ecological mechanisms underlying sympatry in cryptic and pseudocryptic species. As a result, we have only a limited understanding of the factors that govern their distribution along environmental gradients. Here, we used the ribosomal internal transcribed spacer (ITS), 18S rRNA gene, and the RUBISCO large subunit (rbcL) chloroplast gene sequence data together with cell wall morphology to show that estuarine populations of the widespread and common benthic diatom *Navicula phylepta* Kütz. consist of pseudocryptic species. Growth rate measurements in function of salinity showed that *N. phylepta* strains assigned to the different species differed in their tolerance to low salinities (<5 practical salinity units, psu), which was reflected by their different (but widely overlapping) distribution in the Westerschelde estuary (the Netherlands). Multiple regression analyses of the factors determining the abundance of the different species in field samples revealed that, in addition to salinity, sediment type and ammonium concentrations were probably equally important. Our results show that *N. phylepta* sensu lato comprises different species with specialized ecophysiological characteristics rather than generalists with a broad adaptability to different environmental conditions.
Pseudocryptic species in *Navicula phyllepta*

**Introduction**

Polyphasic taxonomic studies based on morphology, molecular data, and reproductive compatibility have led to the recognition that (pseudo)cryptic diversity is widespread among microalgae (Mann 1999, Sáez *et al.* 2003, Slapeta *et al.* 2006, Vanormelingen *et al.* 2008). The term “cryptic diversity” refers to the existence of species that are morphologically indistinguishable, but genetically distinct. In many cases, the results of DNA sequence analyses initiated more detailed morphological studies, which often revealed subtle morphological differences between species, which are then called “pseudocryptic species” (Mann and Evans 2007). In some cases, the recognition of this hidden diversity has resulted in the narrowing of the geographic distributions or ecological amplitudes of species (Rodríguez *et al.* 2005, Casteleyn *et al.* 2008, Kooistra *et al.* 2008). Moreover, an increasing number of studies provide evidence that (pseudo)cryptic microalgal species can also occur in sympatry (Mann *et al.* 2004, Beszteri *et al.* 2005). Their coexistence could be due to fine-tuned niche differentiation, including different preferences or tolerances with respect to small-scale spatial and temporal environmental variation, evolution of different life histories (e.g., in timing of sexual reproduction or the capability of forming resting spores), or differential susceptibility to predators or parasites (Mann 1999). Alternatively, cryptic species may lack obvious ecological differentiation, and random drift and dispersal processes may regulate their relative abundances (Hubbell 2001).

Few studies have addressed the ecological mechanisms underlying sympatry in (pseudo)cryptic species. As a result, there is a limited understanding of which factors govern their distribution along environmental gradients. Ideally, one should combine detailed field studies on the distribution of cryptic or pseudocryptic species with ecophysiological experiments to test whether the presence of a species at a specific site is due to its tolerance to certain environmental conditions or whether other factors (such as biotic interactions or neutral mechanisms) are responsible. However, assessing the distribution of cryptic or pseudocryptic species in nature has by definition been hampered by troublesome or even impossible identifications using standard microscopic approaches (Mann *et al.* 2004).
Recently, it has become possible to use species-specific molecular probes, which holds great promise to monitor (pseudo)cryptic species in natural conditions where they are otherwise difficult to detect (Créach et al. 2006). Laboratory experiments, on the other hand, have shown considerable interclonal variation in the physiological traits of many microalgal morphospecies (Wood and Leatham 1992). As most of these experiments have been conducted before DNA identification methods were available, it is by no means clear whether the ecophysiological differences can be attributed to intraspecific variation or to cryptic species diversity. A study that has correlated ecophysiological differences with genetic diversity was conducted using *Ditylum brighwellii* (Rynearson and Armbrust 2000). Another illustrative example is *Skeletonema costatum* sensu lato (s. l.). This diatom has been thoroughly studied and was considered to be physiologically plastic (Brand 1984), genotypically diverse (Gallagher 1982), and cosmopolitan. Yet, more recent phylogenetic and accurate morphological studies showed that *S. costatum* s. 1. contains several species that seem to be geographically restricted (Sarno et al. 2005, Kooistra et al. 2008). In some cases, the different distributions suggest ecological differentiation between the different species (Sarno et al. 2005, Kooistra et al. 2008).

Estuaries are suitable model systems for studying niche partitioning and changes in species composition along environmental gradients because of the pronounced gradients in, for example, salinity, turbidity, and nutrients. Sibling species of many estuarine taxa have partly overlapping but distinct distributions along the salinity gradient (Bilton et al. 2002). In contrast, some common estuarine diatoms (*N. phyllepta*, *N. salinarum*, *N. gregaria*) apparently occur along whole estuarine gradients, and species exhibit a broad adaptability to different environmental conditions (Krammer and Lange-Bertalot 1986).

*N. phyllepta* is one of the most commonly reported diatoms from brackish and marine sediments worldwide (e.g., Underwood et al. 1998, Clavero et al. 2000, Sabbe et al. 2003). It is particularly abundant in intertidal estuarine and littoral sediments in northwest Europe, where it is often the dominant constituent of microphytobenthic biofilms (Sabbe and Vyverman 1991, Underwood et al. 1998, Haubois et al. 2005). *N. phyllepta* has been reported from a broad range of salinities, from electrolyte-rich freshwaters (Krammer and Lange-Bertalot 1986) to hypersaline environments with salinities up to 75 psu (Clavero et al. 2000). It also shows extensive morphological variability; in particular,
Pseudocryptic species in *Navicula phyllepta* with respect to size and shape (Krammer and Lange-Bertalot 1986, Witkowski *et al.* 2004). Recent sequence analysis of the ribosomal ITS1 region in a number of *N. phyllepta* clones revealed the presence of two distinct ITS1 sequence clusters (Créach *et al.* 2006). A quantitative real-time PCR (qPCR) approach showed that both clades exhibit a distinct but overlapping distribution within the Westerschelde estuary (Créach *et al.* 2006). However, because of the covariation of salinity with many other environmental gradients in the estuary (Ogilvie *et al.* 1997), it is unclear which factors govern the distribution of the two clades of *N. phyllepta*.

Our aim was to further resolve the species boundaries within *N. phyllepta* s. 1. and to determine the ecological preferences of the different cryptic or pseudocryptic species. We first performed a phylogenetic analysis by investigating and comparing variation patterns in three commonly used molecular markers with differing rates of sequence evolution. We complemented this analysis with morphological measurements. We then assessed how (pseudo)cryptic variation was related to ecological characteristics. This was done by using a combined approach in which we (1) determined the growth of a subset of strains in response to different salinities and (2) identified the abiotic factors that govern the field distribution of the different species in the Westerschelde estuary.

**Material and Methods**

**Sampling sites and clonal cultures**

In addition to the 11 *N. phyllepta* strains used by Créach *et al.* (2006) (strain designations beginning with “CCY” and “CO”) from the Westerschelde estuary (the Netherlands) (Fig. 1), the Ems-Dollard estuary on the Dutch-German border, and the Colne estuary (Essex, UK), 10 additional clonal cultures were isolated from sediment samples taken in the Westerschelde estuary (sampling localities and sampling dates in Table 1).

The Westerschelde estuary is the 58 km long Dutch part of the 160 km long Scheldt estuary. The Westerschelde is well mixed and characterized by a complex morphology with tidal channels surrounding
several large intertidal flats and salt marshes. The residence time of the water in the estuary ranges from 1 to 3 months, depending on the season, causing a salinity gradient (ranging from 3 to 32 psu over a distance of 58 km) that is primarily determined by the magnitude of the river discharge and, to a lesser extent, the tidal oscillation, which is of smaller amplitude (Meire et al. 2005). The Ems-Dollard estuary is one of the larger estuaries in the Wadden Sea (Peletier 1996). The brackish zone is ~30 km long and contains large intertidal mudflats. The Colne estuary (UK) is a smaller estuary (~16 km long) and is muddy and highly turbid. The Colne is highly eutrophic, and there are pronounced gradients in nutrient concentrations inversely related to the salinity gradient (Thornton et al. 2002).

Identification of diatoms (cultures and valves in natural samples) as N. phyllepta was based on illustrations of the lectotype shown in plate 32 and figure 5 in Krammer and Lange-Bertalot (1986) and in figure 28 in Cox (1995). The salinities (average ± SD, based on measurements between April 2002 and September 2003) at the four Westerschelde sampling sites, Appelzak (51°22'38" N, 4°14'32" E), Bath (51°23'58" N, 4°12'36" E), Biezelingsche Ham (51°26'50" N, 3°55'33" E) and Paulina Schor (51°20'58" N, 3°43'37" E), were 8.8 ± 4.7 (n = 18), 13.6 ± 3.8 (n = 9), 20.0 ± 3.8 (n = 18), and 22.7 ± 6.3 (n = 18) psu, respectively.

![Figure 1. Map of the Westerschelde estuary (the Netherlands) with the different sampling locations from which Navicula phyllepta strains were isolated. PS, Paulina Schor; BH, Biezelingsche Ham; Ba, Bath; AZ, Appelzak. The oligohaline zone ranges from 0.5 to 5 psu; the mesohaline zone, from 5 to 18 psu; and the polyhaline zone, from 18 to 30 psu.](image-url)
Clonal cultures were established as described in Chepurnov et al. (2002) and grown in artificial seawater (Ultramarine Sea Salt; Waterlife Research LTD, Middlesex, UK) with a salinity of 30 ± 1 psu, enriched with F/2 nutrients (Guillard 1975). The possibility of a bias to clonal cultures adapted to 30 psu cannot be fully excluded, but it is considered unlikely because estuarine benthic diatoms are known for their ability to grow at a wide range of salinities. Moreover, we did not find different morphotypes of *N. phyllepta* in the field compared to the isolated strains (see Results). The cultures were kept in 24-well plates (Greiner Bio-One, Frickenhausen, Germany) at 18 ± 0.3°C with a 12:12 light:dark (L:D) period and 25-30 μmol photons m⁻² s⁻¹ from cool-white fluorescent tubes (Philips, Eindhoven, the Netherlands, TLD 18W). The cultures were transferred every 2 weeks to fresh medium.

**DNA amplification and sequencing**

Three DNA regions with differing rates of sequence evolution were selected for sequencing, that is, the ITS region (consisting of ITS1, 5.8S rRNA gene, and ITS2), 18S rRNA gene (which is transcribed into the ribosomal SSU), and a 457 bp fragment of the chloroplast RUBISCO large subunit gene (*rbcL*) (Table 1). The non-coding ribosomal ITS region is highly variable and is often used to investigate variation within populations. The *rbcL* and 18S rRNA genes are more slowly evolving. In raphid diatoms, *rbcL* evolves slightly faster than the 18S rRNA gene (Evans et al. 2008).

Cells for DNA extraction were harvested from exponentially growing cultures and pelleted by centrifugation (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany, 4K15 centrifuge). For the amplification and sequencing of the 18S rRNA gene and the ribosomal ITS region, DNA was extracted using the bead-beating method with phenol extraction and ethanol precipitation as described by Zwart et al. (1998). For *rbcL* amplification and sequencing, DNA was extracted using a commercial kit (Mo Bio Lab. Inc., Carlsbad, CA, USA). The ITS of 11 strains (accession numbers of these strains in Table 1 are not in bold) was cloned and sequenced earlier (Créach et al. 2006). The PCR products of the ITS were cloned in PCR II-TOPO vector TA cloning kit (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. For nine strains (Table 1), two to four clones were sequenced and analyzed. For the other nine strains (strain designations starting with “AP” or
Chapter 2

BA"), the ribosomal ITS1-5.8S-ITS2 region was amplified by PCR using the universal primers ITS4 (reverse primer located at the beginning of the 28S rRNA gene, White et al. 1990) and 1800F (forward primer located at the end of the 18S rRNA gene, Friedl 1996). The PCR reaction mixture contained 1 μL of template DNA, dNTPs at 0.2 mM, each primer at 0.5 μM, 5 μL 10X PCR buffer (100 mM Tris-HCl [pH 9], 500 mM KCl), and 2.5 U Taq Polymerase (Qiagen, Hilden, Germany) and was adjusted to a final volume of 50 μL with sterile water (Sigma, St. Louis, MO, USA). PCR reaction conditions consisted of an initial preheating step of 3 min at 94°C followed by 40 cycles of 94°C denaturation for 1 min, 55°C annealing for 2 min, and 72°C extension for 1 min. Finally, there was a 72°C extension for 5 min. Sequencing was performed using a Perkin-Elmer ABI Prism 377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequencing primers were DITS2 and DITS3 (Zechman et al. 1994), and sequence coverage was forward and reverse.

For the 18S rRNA gene, forward primers were DSSU4 (5'-AACCTGGTTGATTCTGCCAGTAG-3'), DSSU550 (5'-AAGTCTGGTCGCCAGGCC-3'), and DSSU1119 (5'-GGCTGAAACTTAAAGAAATG-3'). Reverse primers were DSSU376 (5'-TCTCAGGCTCCCTCTCG-3'), DSSU1180 (5'-TCCACCAACTAAGAACGGCC-3'), DSSU1613 (5'-GTACAAAGGGGACGGAAGCT-3'), and DSSU1860 (5'-CTCGCAAGTTCACCTACGGAAACC-3'). For the PCR, the reaction mixture contained 1–5 μL of template DNA, dNTPs at 0.2 mM, primers at 1 μM each, 2.5 U Taq polymerase, and PCR buffer and was adjusted to a total volume of 50 μL with sterile water. PCR reaction conditions consisted of an initial 94°C denaturation during 5 min followed by 40 cycles of 94°C denaturation for 30 s, 55°C annealing for 2 min, and 72°C extension for 2 min. The 18S rRNA gene sequences were obtained by direct sequencing from the PCR product using the ABI 3100 prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequence coverage was forward and reverse.

A 554 bp fragment of the rbcL gene, which includes the functional site of the enzyme, was amplified in a PCR with a degenerate primer pair as described in Wawrik et al. (2002). PCR mixtures (50 μL) contained 1 μL of template DNA, primers at 0.5 μM each, dNTPs at 0.2 mM, 1.5 mM MgCl2, 2.5 U of Taq DNA polymerase, and PCR buffer (Qiagen). PCR reaction cycles were as described in Wawrik et al. (2002). Sequences were obtained with the ABI 3100 prism BigDye Terminator.
Pseudocryptic species in *Navicula phyllepta*

Cycle Sequencing Ready Reaction Kit. GenBank accession numbers of all *N. phyllepta* sequences are listed in Table 1. The *rbcL* fragments are only 457 bp long, lacking the first ~45 bp at either end as the result of unresolved parts at the beginning and the end of the sequence chromatograms.

**Phylogenetic analyses**

Sequences of each molecular marker (ribosomal ITS, *rbcL*, 18S rRNA gene) were edited separately using BioNumerics version 3.5 (Applied Maths, Kortrijk, Belgium) and automatically aligned using ClustalX (Thompson et al. 1997). The resulting alignment was manually corrected where necessary. Phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford 2001) and, for Bayesian inference (BI), MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). Uncorrected *P* distances (Nei and Kumar 2000) were calculated as measures of genetic distance between the sequences in each alignment. Phylogenetic relationships were assessed separately using maximum parsimony (MP) and Bayesian Inference (BI) on the ITS1, the ITS2, the complete ribosomal ITS, the 18S rRNA gene, and *rbcL* alignments. For the ITS1 and ITS alignments, the first 36 positions were omitted because this part was lacking for two of the sequences, due to unresolved parts at the beginning of the sequence chromatograms. No outgroup was specified. For MP analysis, the sites were treated unweighted. Gaps were treated as missing data. MP trees were inferred using heuristic searches with random stepwise additions of taxa, repeated 100 times, and using the tree bisection-reconnection branch-swapping algorithm. Bootstrap values were assessed from 1,000 replicates. For the BI, we used MrModeltest 2.3 (Nylander 2004) to calculate the best-fitting model for the given data matrix using the AIC (Akaike information criterion). Likelihoods for 24 models of evolution were calculated using PAUP*, and the command file was provided to MrModeltest 2.3. The selected best-fitting models were K80 + I (for the ITS), K80 (for ITS1), HKY+1(for ITS2), GTR + I + G (for 18S rRNA gene), and GTR + G (for *rbcL*). No initial values were assigned to the model parameters. Two runs of four Markov chains (one cold and three heated) were run for three million generations and sampled every 100 generations. This yielded a posterior probability distribution of 30,001 trees. After exclusion of 5,000 “burn-in” trees, posterior probabilities were calculated by constructing a 50% majority-rule consensus tree.
Table 1. *N. phyllepta* strains used in this study together with place and date of isolation, GenBank accession (acc) numbers of the corresponding ribosomal ITS, 18S rRNA gene and *rbcL* sequences, morphometric measurements (mean ± SD) and assignment to the different morphotypes, physiotypes and phylogenetic clades. Strains from clade B are indicated by grey shading. Accession numbers of sequences new

<table>
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Mean morphometric features clade A

| CCY 0220     | N100      | Sep '02        | Appelreek             | DQ103560     | EU93832     |
| CCY 0201     | N100      | Jan '02        | Bezdolnecice Hamara   | DQ103546     | EU938309    | EU93831  |
| CCY 0212     | N100      | Apr '02        | Paulina Schor         | DQ103548     | FJ624241    | EU93832  |
| CCY 0213     | N100      | Apr '02        | Paulina Schor         | DQ103549     | FJ624241    | EU93832  |
| CCY 0227     | N100      | Sep '02        | Paulina Schor         | DQ103583     | FJ624253    | EU93831  |
| CCY 0226     | N100      | Apr '08        | Kest Devil (NL)       | DQ103551     | EU938311    | EU93831  |

Mean morphometric features clade B

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Pseudocryptic species in *Navicula phylepta*
to this study are in bold. Morphotype: "S": Narrow valves with high stria density, "R": Wide valves and low stria density. Growth 2 psu: ability to grow at 2 psu: "+" = yes, "-" = no and cells died, "±" = no growth but cells survived. PAE: Research Group Protistology and Aquatic Ecology, Ghent, Belgium. NIOO-CEME: Yerseke, The Netherlands. Essex: Dept. Biol. Univ. Essex, UK. "nm" stands for 'not measured'.

<table>
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<th>Striae (No. 10 µm)</th>
<th>Morphotype</th>
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<td>A</td>
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Valve morphology

For morphological observations of the siliceous cell wall, all clonal cultures and three natural samples (Appelzak, Biezelingsche Ham, and Paulina Schor) were oxidized using hydrogen peroxide (15% [v/v]) and acetic acid (50% [v/v]) and repeatedly washed with distilled water before being mounted in Naphrax (PhycoTech, St. Joseph, MI, USA). LM was carried out using a Zeiss Axioplan 2 microscope (Zeiss Gruppe, Jena, Germany) equipped with a digital camera (Zeiss Axiocam MRm). Morphometric measurements on digital images were made using Image J software (Abramoff et al. 2004). Length, width, and stria density of 20 frustules from each strain and 100 from each natural sample were measured. Voucher specimens of cleaned material of the original natural sample and the clonal cultures are kept at the Laboratory of Protistology and Aquatic Ecology, Ghent University, Belgium (voucher specimens PAE00101–PAE00115).

Ecophysiology and natural distribution

To assess growth performance at different salinities, eight *N. phyllepta* strains (CCY0201 from Biezelingsche Ham; CCY0212 and CCY0213 from Paulina Schor; and BA-04-01, BA-04-02, BA-04-04, and BA-04-05 from Bath; and CCY0221 from Appelzak) were grown in triplicate at eight different salinities (0, 0.5, 1, 2, 5, 10, 20, and 30 psu). Suspended cells from stock cultures grown at 30 psu until late exponential phase were transferred into wells of flat-bottom 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany). Wells were inoculated at a cell density of ~3,000 cells mL⁻¹, resulting in an initial density of ~29 cells mm⁻². One hour after the inoculation, when most of the cells had settled at the bottom, the culture medium was gently pipetted off and immediately replaced by fresh medium at the desired salinity. Growth was not acclimated to the different salinities prior the experiments. A representative growth curve is shown in Figure 2.
Figure 2. Growth curve of *Navicula phyllepta* strain CCY0221 at 10 psu. Error bars represent standard deviations.

There were some clear signs of osmotic stress and cell lysis at 0, 0.5, and 1 psu, especially in the case of strains belonging to clade B. Culture media were prepared by adding F/2 nutrients (Guillard 1975) and salts (Ultramarine Sea Salt; Waterlife Research LTD, Middlesex, UK) to distilled water up to the required salinity. The culture media were filtered through glass fiber filters (0.7 μm pore size, Whatman, Maidstone, England, GFF) and autoclaved. Growth conditions were identical to those described above for maintaining the stock cultures. Cell densities of each replicate were monitored daily using an inverted microscope (Reichert PV 624; Reichert-Jung, Vienna, Austria) by counting a minimum of 400 cells per replicate.

Growth rate during the exponential phase (4–5 d) was calculated as the slope of the linear regression of log2-transformed cell densities versus time for individual cultures (Underwood and Provot 2000). Because the results of these experiments indicated that the strains differed mainly in their ability to grow at low salinities (0.5–5 psu), six additional strains (AP-02-05 from Appelzak; BA-04-06, BA-04-07, BA-04-08, and BA-04-09 from Bath; and CO-04-01 from the Colne estuary [UK]) were grown only at 2 and 5 psu, and qualitative observations were made on their survival and growth. Cultures were followed during 2 weeks, a period during which healthy cultures reached stationary phase.

The relationship between the environmental variables and the distribution of cells belonging to clades A and B in the Westerschelde estuary was explored using stepwise forward multiple regression as implemented in Statistica version 6.0 for Windows (StatSoft Inc., Tulsa, OK, USA). This analysis was performed displaying results at each step, with an F to enter set at 1.00 and an F to remove set at 0; tolerance was
set at 0.0001, and the intercept was included in the model. Only significant variables were included in the model. The Pearson's residuals are given in supplementary figure 1. Standardized residuals were tested for normality using the Shapiro Wilk's W test. All locations (Appeizak, Biezelingsche Ham, and Paulina Schor) were sampled nine times, spanning two consecutive years. At each location, highshore and midshore stations were selected on the exposed mudflats. Samples were taken in April 2002, May 2002, September 2002, February 2003, March 2003, April 2003, May 2003, July 2003, and September 2003. Samples were taken during low tide. The strains with designation starting with "CCY" were taken during this sampling campaign. The distribution data used for the stepwise forward multiple regression were obtained from Créach et al. (2006) who conducted a qPCR to determine the abundance of clades A and B in the samples taken during the above-mentioned campaign.

The environmental parameters measured during this sampling campaign were obtained from Sahni et al. (2007) and included ammonium, nitrite, nitrate, phosphate, salinity, organic carbon content, organic nitrogen content, C:N ratio, temperature, irradiance, water content, mean grain size, silt content, and height of the sediment. Surface sediment samples (upper 2mm) were collected using a contact corer. Total organic carbon (TOC) was measured using an elemental analyzer (Elementar Analysensysteme, Hanau, Germany). Organic nitrogen content, nitrite, nitrate, ammonium, and phosphate were measured using standard colorimetric techniques (Grasshoff 1976) on a SKALAR SA 4000 segmented flow analyzer (Skalar Analytical B.V., Breda, the Netherlands). Salinity was measured using a titration method with SAC 80 (Radiometer, Copenhagen, Denmark). Sediment grain size and silt content of the sediment were determined by granulometric analysis using a laser diffraction analyzer (Malvern Mastersizer 2000; Malvern Instruments Limited, Malvern, UK). The percentage of water was determined by the loss of weight of the sample after 48 h of freeze-drying. The shore heights of the stations were determined by reference to a digital elevation model of the estuary and confirmed by direct observation of the timing of emersion and immersion periods. Sediment surface temperatures were measured with an electronic thermometer at each sampling occasion. The mean irradiance at all sites was recorded at hourly intervals during 2002 and 2003 using a Li-Cor Li-192 sensor (Li-Cor, Lincoln, NE, USA).
Pseudocryptic species in *Navicula phyllepta*

**Results**

*Molecular phylogenies.* Phylogenetic analyses performed on the ITS sequences of the strains identified morphologically as *N. phyllepta* revealed two distinct clades (Fig. 3A) possessing intraclade *P* distances not higher than 0.0148 (12 nucleotide differences) and interclade distances between 0.0762 and 0.0898 (78 to 92 nucleotide differences). Both clades were supported by high to maximal MP bootstrap and BI posterior probability values. These two clades are referred to as clades A and B (see Fig. 3A). The strains collected from the Colne estuary (CO-04-01) and Ems-Dollard (CCY9804) belonged to clades A and B, respectively. Intraclonal ITS variation was very low (*P* distances not higher than 0.010 and 12 nucleotide differences) (Fig. 3).

Phylogenetic analyses performed on the ITS1 and ITS2 regions generated nearly identical trees supported by high to maximal MP bootstrap and BI posterior probability values (not shown). The ITS1 was more variable than ITS2, with interclade uncorrected *P* distances between 0.119 and 0.141, and 43 to 59 nucleotide differences, compared to interclade distances between 0.064 and 0.084, and 25 to 33 nucleotide differences for the ITS2. The partial *rbcL* (which includes the functional site of the enzyme, Wawrik et al. 2002) and 18S rRNA gene phylogenies also supported the distinction of clades A and B (Fig. 3, B and C, results summarized in Table 1). The number of nucleotide differences among the partial *rbcL* sequences within the clades was 0 or 1 (*P* distances 0.000–0.002), while sequences from different clades (A and B) differed at 8 to 9 positions (*P* distances 0.018–0.021). Eight of the nine differences were in the third codon position and did not result in amino-acid differences. One nucleotide difference was in the first codon position and resulted in a change of amino acid. In the 18S rRNA gene (the first 129 and last 93 nucleotides are missing), the two clades had two nucleotide differences (*P* distance 0.001), while sequences within each clade were identical (except strain BA-04-07, which differed at one position from the other strains of clade A). The 18S rRNA gene sequence of a *Navicula* sp. strain (AT-145.08) collected in a brackish lagoon of the Baltic Sea by Bruder and Medlin (2008) belonged to clade A, indicating a wider distribution of this clade. Both *rbcL* and 18S rRNA gene phylogenies suggest that *Seminavis* cf. *robusta* and *Pseudogomphonema* are close relatives of *N. phylepta*, closer than other *Navicula* species, such as *N. cryptotenella* and *N. reinhardtii*. 
Figure 3. Phylogeny obtained from Bayesian inference (BI) of (A) ribosomal ITS, (B) rbcL, and (C) 18S rRNA gene. The trees shown are the 50% majority-rule consensus tree based on BI analysis. Bootstrap values >50% (MP) or posterior probabilities >0.5 (BI) are indicated on the respective nodes. Intraclonal sequence variants are indicated by a different letter behind the strain name. Scale bars represent one substitution in 100 nucleotides (A) or one substitution in 10 nucleotides (B and C). ITS, internal transcribed spacer; MP, maximum parsimony.
Valve morphology

LM photographs of *Navicula phyllepta* valves are shown in Figure 4. While there were no obvious differences between the species in general valve features (valve outline, striation pattern, etc.), they differed in their morphometry (Table 1 and Fig. 5). Based on a combination of valve width and stria density, there was a clear distinction between strains of the clades A and B. Strains belonging to clade A were narrow (4.2–5.5 μm wide) and had a high stria density (19.5–24 striae in 10 μm) (Fig. 5A). Strains belonging to clade B had wider valves (5.5–7.5 μm) and a lower stria density (16–20 striae in 10 μm) (Fig. 5A). When strains with the same length belonging to the different clades (e.g., CCY0221 and BA-04-07 of clade A and CCY0212 and CCY9804 of clade B) were compared, it was clear that the differences in width and stria density were maintained when cells belonging to different clades had the same length.

![Figure 4. LM photographs of *Navicula phyllepta*. (A) Strain BA-04-01 (clade A). (B) Strain CCY0212 (clade B). Scale bar, 10 μm.](image)
Morphometric analysis of *N. phyllepta* valves from the samples from the Westerschelde (locations Appelzak, Biezelingsche Ham, and Paulina Schor) showed the presence of the same two groups based on differences in stria density and valve width (Fig. 5, B and C). The natural samples were composed of a group with narrow frustules (4.3–5.5 μm) having higher stria densities (20–23 striae in 10 μm) and a group with broader frustules (5.4–8 μm) and lower stria densities (16–20 in 10 μm). Both groups also differed in valve length, with the maximal length for the broader group being 31–32 μm in the field, while that of the narrower group was 21–22 μm. The minimal valve length in the field was 15–16 μm and 11–12 μm, respectively (Fig. 5C). With decreasing length of the cells, the width also decreased, but the length-associated differences were smaller than the differences between the different groups (Fig. 5C). Unfortunately, we failed to initiate auxosporulation in culture, and therefore we were unable to determine the maximum cell length for the two clades. The minimum size of all strains in culture was more or less the same, ~9–10 μm. When the cell reached this "critical" size, the cultures ceased to grow and subsequently died.
Figure 5. Scatter plots of (A) valve width versus stria density of *Navicula phyllepta* strains, (B) valve width versus stria density of three natural samples from the Westerschelde estuary, and (C) valve length versus valve width of three natural samples from the Westerschelde estuary.
Ecophysiology and distribution pattern

Irrespective of clade affinity, all strains tested grew well in a broad range of salinities but were unable to tolerate freshwater conditions (Fig. 6). However, strains from the two clades differed considerably in their tolerance to low salinities (0.5–2 psu). Strains belonging to clade A were able to grow at salinities as low as 0.5 psu, while monoclonal cultures belonging to clade B showed growth only at 5 psu or higher and died at salinities below 5 psu. There was one exception: cells of strain BA-04-05 (belonging to clade A) grown at 0.5–2 psu did not divide but were still motile and contained healthy-looking chloroplasts even after 10 d. Qualitative testing of six additional strains for growth at two salinities (2 and 5 psu) showed that all six clonal cultures belonging to clade A were able to grow at 2 psu (Table 1). The strain originating from the Colne estuary (UK) showed the same response to salinity as the other strains belonging to clade A.

Figure 6. Growth rate of eight Navicula phyllepta strains grown in triplicate at eight different salinities. Strains shown in black belong to clade A; strains in gray, to clade B.
Pseudocryptic species in *Navicula phyllepta*

The abundances of cells belonging to clade A in the Westerschelde estuary were negatively correlated with salinity ($r = -0.33$, $P = 0.015$), while no significant correlation between salinity and the occurrence of cells belonging to clade B was found ($r = -0.0129$, $P = 0.926$). In a multiple regression model explaining the abundances of cells belonging to clades A and B in the Westerschelde estuary, organic nitrogen content, irradiance, and salinity were selected for clade A (Table 2, Fig. 7). Together, these variables explained a highly significant [$F(3,5) = 14.03$, $P$ value < 0.0001] 42.4% ($R^2$ adj.) of the variation in the abundance of cells belonging to clade A. Ammonium concentration, silt content, irradiance, temperature, and nitrite were selected for clade B (Table 2, Fig. 7). Together, these variables explained a highly significant [$F(5,48) = 17.693$, $P$ value < 0.0001] 61.2% ($R^2$ adj.) of the variation in the abundance of cells belonging to clade B.

![Figure 7](image_url)

**Figure 7.** Scatter plots of the log-transformed abundances of *Navicula phyllepta* clade A or B against some of the environmental factors selected in a stepwise forward multiple regression model. (A) Organic nitrogen (OrgN) content versus abundances of cells belonging to clade A. (B) Pore water salinity versus abundances of cells belonging to clade A. (C) Silt content versus abundances of cells belonging to clade B. (D) Ammonium concentration versus abundances of cells belonging to clade B. Significant correlations ($P < 0.05$) are indicated by a regression line.
Chapter 2

Table 2. Results of stepwise multiple regression for the abundances of cells belonging to clades A and B in the Westerschelde estuary. Estimated regression coefficients (β) and their standard errors (S.E.) and associated significance level (P) for each variable. Only significant variables are shown for the different clades.

<table>
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<tr>
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<td>P-value</td>
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<td>S.E.</td>
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Discussion and conclusions

On the basis of three molecular markers (viz. ribosomal ITS, 18S rRNA gene, and rbcL) with different rates of sequence evolution, it was shown that *N. phyllepta s. l.* consists of at least two clearly distinct clades with low intraclade sequence divergence compared to the interclade divergence. These results confirm and strengthen the conclusions drawn by Créach et al. (2006) who showed the existence of two ITS1 sequence clusters. Créach et al. (2006) also found that the clades A and B differed in the number of copies of the ribosomal operon as well as a 4-fold difference in the cellular DNA content. The congruence of the different molecular markers, the low intraclade variation, the low intracloal variation, and the differences in DNA content suggest the presence of intrinsic barriers to gene flow between these sympatric species, as has been shown in some other microalgae (e.g., Coleman 2000, Behnke et al. 2004). Repeated attempts to set intercloal mating tests within and between the clades were unsuccessful. We were unable to initiate sexual reproduction despite using methods that have been proved successful for many other diatom species (see, e.g., Chepurnov et al. 2004). The average cell size in the *N. phyllepta* cultures gradually decreased, and cultures were eventually lost when they reached the critical minimum size. We therefore conclude that a mechanism must exist that restores the cell size, and it seems
reasonable to assume that this is associated with sexual reproduction and auxospore formation, as is the case in most diatoms.

The differentiation in molecular markers was associated with subtle but discrete differences in valve morphology between strains of clades A and B, which should therefore be referred to as pseudocryptic. There was a subtle but clear morphological distinction based on a combination of valve width and stria density. Both width and stria density were dependent on length and change during the life cycle, as is the case in other diatoms (Mann 1999, Mann et al. 1999). However, these length-associated differences are smaller than the differences between the different clades; hence, there is no overlap in morphology. The pseudocryptic species are probably sibling as they are more closely related to each other than to other Navicula species, although taxon coverage in this genus is fragmentary. Several molecular studies have now demonstrated that protist species traditionally defined on the basis of morphology often consist of two or more genetically distinct groups that often occur in sympatry (e.g., Slapeta et al. 2006, Vanormelingen et al. 2008). Moreover, evidence of reproductive isolation of such sympatric cryptic species was found for several protists (Coleman 2000, Amato et al. 2007, Vanormelingen et al. 2008). In most cases, it is unknown whether these seemingly similar species differ in their biology or whether they are functionally equivalent. As a consequence, we know little about the mechanisms that promote the coexistence of cryptic protist species. The limited available data suggest or show differentiation between cryptic species for susceptibility to different parasitic fungi (Mann 1999) and light requirements (Rodriguez et al. 2005), which might help to explain their sympatric occurrence. There is evidence that different tolerances to salinity may contribute to niche differentiation between allopatric cryptic species (Koch and Ekelund 2005, Lowe et al. 2005).

For N. phylepta, the sibling species were differentiated in terms of growth at different salinities, and this corresponded with their distribution in the Schelde estuary. As previously shown for other estuarine diatoms (Admiraal 1977, Brand 1984, Clavero et al. 2000), all strains grew well over a wide range of salinities, including the average salinities at the different sampling stations (8.8–22.7 psu), seemingly suggesting that the distribution is not the result of physiological constraints. However, strains of clade B were not able to grow at low (0.5–5 psu) salinities, in contrast to the strains belonging to clade A. Such small differences in physiological tolerances may influence species
interactions and consequently their distribution in the field (De Jong and Admiraal 1984). In particular, the different tolerances to low salinities could be important during heavy rainfall when the salinities of the sediment top layer often drop to almost freshwater conditions (Admiraal 1977, Coull 1999). This tolerance difference would give clade A organisms a competitive advantage in the oligohaline and mesohaline parts of the estuary. Thus, despite the wide salinity range in which the species were able to grow, their tolerance to low salinities may nevertheless determine their abundance. In line with this, the abundance of clade A organisms in the Westerschelde estuary was indeed positively associated with low salinities, while clade B organisms reached their highest abundances at higher salinities (>15 psu). However, this difference was not significant because of the large interannual variation (clade B only bloomed in spring of the first sampling year). From Figure 6, it is clear that strains of clade A have higher growth rates in culture than those of clade B at any given salinity and that their optimal salinity is ~20 psu. This finding suggests that clade A should be able to outcompete clade B. However, making predictions about growth rates and competitive ability in the field based on absolute growth rates obtained in the laboratory is difficult. For example, *N. salinarum* displays optimal growth rates at 30 psu (Underwood and Provot 2000) but occurs only in brackish environments where it outcompetes other species because of its tolerance to low salinities and high ammonium and sulfide concentrations (Peletier 1996, Underwood and Provot 2000). This phenomenon suggests that tolerance, rather than the growth rate, determines the ecological success of an organism and would explain species distribution.

Salinity is an important factor influencing the distribution of the two clades within *N. phyllepta* (and of other diatoms, Sabbe and Vyverman 1991, Underwood *et al.* 1998). However, other environmental variables may be equally important, as is suggested by the multiple regression model. The model shows that, independent of each other, organic nitrogen content, irradiance, and low salinity positively influenced the abundance of clade A. The organic nitrogen content had the strongest contribution to the model and is significantly correlated with silt content ($r = 0.6521, P = 0.000$), ammonium concentrations ($r = 0.5225, P = 0.000$), phosphate concentrations ($r = 0.6479, P = 0.000$), water content ($r = 0.8114, P = 0.000$), and organic carbon content ($r = 0.9095, P = 0.000$) (All correlations are given in supplementary table 1). Thus, clade A typically blooms in spring and summer in the mesohaline parts of the
Pseudocryptic species in *Navicula phylepta*

estuary on silty sediments (70%-90% silt) with high organic matter and water content and high nutrient concentrations. Clade B was positively influenced by high ammonium concentrations and lower silt content. This clade reached its highest abundances at sediments with lower silt content (30%-70%) and with high ammonium concentrations (200-450 μM).

Nitrite, which was generally higher in silty sediments, also negatively influenced its occurrence. Furthermore, clade B was positively influenced by irradiance, but negatively by temperature, suggesting that this species peaks in spring, which could be due to temperature tolerances but might also be due to high grazing pressures in late spring and summer (Sahan et al. 2007). Salinity, sediment type, organic matter content, ammonium concentrations, and weather conditions thus differently influence the spatial and temporal distribution of both clades of *N. phylepta*. Several studies show that these variables strongly affect the species composition of microalgal biofilms on intertidal mudflats (Peletier 1996, Underwood et al. 1998, Thornton et al. 2002). Ammonium can have positive effects on the growth and distribution of benthic algae as it is an easily assimilated source of nitrogen (Underwood and Provot 2000). However, high ammonium concentrations may have detrimental effects on benthic algae, especially on *N. phylepta* (Peletier 1996, Underwood and Provot 2000). The very high ammonium concentrations (400-1,000 μM) necessary to cause detrimental effects were (almost) not encountered in the Westerschelde estuary, and a negative effect was therefore not expected.

Our conclusion that *N. phylepta* consists of different pseudocryptic species possessing different preferences for salinity and other environmental factors may explain the puzzling pattern of field observations of *N. phylepta*. This “morphospecies” was reported in different estuaries (Underwood and Barnett 2006) at salinities ranging from 6 to 35 psu (Cox 1995, Snoeijis and Potapova 1995, Peletier 1996, Thornton et al. 2002). These observations can probably be attributed to the occurrence of different pseudocryptic species with distinct environmental preferences, rather than a single generalist species that exhibits a broad adaptability to different salinity conditions. This phenomenon is found for several coastal and estuarine macroscopic eukaryote morphospecies displaying substantial morphological and physiological variation. Detailed study revealed that these morphospecies consist of several (pseudo)cryptic species with different habitat requirements (Knowlton 1993). This finding has been shown, for instance,
in nematodes (Derycke et al. 2005), in copepods, and for many other invertebrates (Bilton et al. 2002). Finlay (2002) argues that because microbial eukaryotes are able to tolerate or adapt to a wide range of ecologically important factors, there will be few physiological “species” within microbial morphospecies. Our findings oppose the view that microbial species are ecological generalists. We show that there may be a strong discrepancy between the measured theoretical niche (which can be very broad) and the realized niche in the field. We further demonstrated that the morphospecies *N. phylepta* consists of at least two distinct clades, and that despite their genetically and evolutionary relatedness, these clades display important physiological differences and are influenced differently by environmental gradients, resulting in distinct distributions along the Westerschelde estuary.

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Pseudocryptic species in *Navicula phyllepta*


Koch, T. A. & Ekelund, F. 2005. Strains of the heterotrophic flagellate *Bodo designis* from different environments vary considerably with


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Chapter 2


Supplementary material

Supplementary figure 1. Pearson’s residuals for the stepwise multiple regressions shown in table 2.
Chapter 3

Abstract

How similar are closely related lineages of microalgae in their ecological niches? Can thermal niches evolve fast on an evolutionary timescale resulting in regular switches between climates or are they instead highly conserved? To address these questions we composed a set of closely related strains of the globally distributed diatom genus *Cylindrotheca*. We collected strains from a wide range of marine habitats, from coastal plankton to sea ice and intertidal mudflats. We first inferred the evolutionary relationships of these strains using a multi-locus DNA dataset and obtained a well-resolved phylogeny. We then determined temperature preferences of closely related lineages in laboratory experiments. Combining the molecular phylogeny with the thermal niches of lineages revealed a very weak phylogenetic signal in thermal niche characteristics. This indicates that closely related species tend to differ more in thermal niche than expected by a random walk model. This seems to be caused by a combination of adaptive evolution and frequent shifts in environments in related lineages.
Introduction

To what extent closely related species are similar in their ecological niches is a major and recurring theme in evolutionary biology and ecology (Harvey & Pagel, 1991, Losos, 2008, Wiens et al., 2010). Two extremes along a continuum of niche evolution can be distinguished: phylogenetic niche conservatism and adaptive radiation (Ackerly, 2009). Phylogenetic niche conservatism (PNC) is the tendency of species to retain aspects of their fundamental niche over time (Wiens & Graham, 2005). Conservative evolution of ecological traits can arise from different processes such as stabilizing selection (Ackerly, 2003), insufficient genetic variation (Bradshaw, 1991) and homogenizing gene flow counteracting local niche adaptation (Holt & Gomulkiewicz, 1997). While several studies have demonstrated PNC in a broad range of organisms (e.g. Ackerly, 2004, Kozak & Wiens, 2006, Wiens et al., 2006, Verbruggen et al., 2009, Bahl et al., 2011), others find no or only weak evidence for PNC and instead report no or only little relation between phylogenetic relatedness and niche similarity (Rice et al., 2003, Cavender-Bares et al., 2004, Graham et al., 2004), for instance in the case of adaptive radiation (Losos et al., 2003). Adaptive radiation can be defined as "the evolutionary divergence of members of a single phylogenetic line into a variety of different adaptive forms"; usually this divergence occurs over a relatively short geological time span (Futuyma, 1998). Given the paucity of data, it is at present not known how important PNC is, and more importantly to what degree organismal or environmental features influence the incidence of PNC.

Ideally, the combination of robust phylogenetic information and information on niche preferences allows estimating how fast ecological divergence evolves between related species (Cooper et al., 2010). Niche information can be obtained either by ecological niche modelling or by ecophysiological experiments. Niche modelling techniques are based on data on the current distribution of a species and the range of environmental conditions it occupies (the realized niche), which is typically smaller than the suit of environments a species can potentially inhabit (the fundamental niche) (Colwell & Rangel, 2009). This, together with the choice of modelling techniques can influence the patterns inferred from a phylogenetic comparative analysis. Ecophysiological
experiments allow defining the fundamental niche of organisms for the environmental variable of interest.

Our views on evolutionary niche dynamics are biased towards macroscopic and often eye-catching species mainly from terrestrial communities; a selection often guided by natural history observations on striking ecological differentiation (Ackerly, 2009). This clade selection disregards the microbial world. Little is known about evolutionary niche dynamics in marine microbial species (Palumbi, 1994). Marine microbial populations are often believed to lack geographical barriers to gene flow (Cermeño & Falkowski, 2009, but see Casteleyn et al., 2010) which would frequently lead to the dispersal of large numbers of cells into unfavourable environments. It has been hypothesized that marine communities are characterized by a widespread occurrence of ecological speciation, i.e. reproductive isolation caused by divergent natural selection (e.g. Ingram, 2011), but the importance of this mechanism is unknown in marine microbial communities.

In this study we focus on the globally distributed diatom genus Cylindrotheca. Species of this genus are common in a wide range of coastal habitats, from coastal plankton to sea ice and intertidal mudflats. Our goal was to determine the evolutionary thermal niche dynamics of marine Cylindrotheca species. Therefore, we first determined the phylogenetic relationships between Cylindrotheca lineages and experimentally quantified their fundamental temperature niches. We then integrated the fundamental thermal niche and the phylogenetic information to infer temperature niche evolution by calculating the phylogenetic signal in the available data.

Materials and Methods

Taxon sampling

We collected a set of 40 strains belonging to Cylindrotheca cf. closterium and C. cf. fusiformis (Table 1). Twenty-two strains were newly isolated from marine and brackish sediment and plankton samples (sampling localities in Table 1 and Fig. 1). Diatoms were harvested from sediments using the lens tissue technique (Eaton & Moss, 1966); migratory
microalgae were collected by placing a piece of lens tissue on top of the sediment followed by a coverslip on the tissue, which was transferred to autoclaved seawater after 3 hours of incubation at low light. We established monoclonal, non-axenic cultures by isolating single cells by micropipette and subsequent culturing in sterile filtered (0.2 μm) seawater (33 psu) enriched with f/2 nutrients (Guillard, 1975). The other 18 strains were obtained from the Provasoli-Guillard National Centre for Culture of Marine Phytoplankton, U.S. (CCMP); the Commonwealth Scientific and Industrial Research Organization (CSIRO) collection of living microalgae (Australia), and the culture collection of algae and protozoa (CCAP), UK. Strains PT01, SP01, GB01 and CIM222 were kindly provided by J. Serodio, I. Moreno Garrido and J. Taylor and M. Pfannkuchen.

Figure 1. Geographic distribution of Cylindrotheca strains.
Table 1. List of *Cylindrotheca* strains used in this study. C.f. = *Cylindrotheca fusiformis*, C.c. = *Cylindrotheca closterium*.

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<th>Latitude/Longitude</th>
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All cultures were kept in 24-well plates (Greiner Bio-One, Frickenhausen, Germany) at 18 ± 0.3°C with a 16:8 light:dark period and 50 μmol photons m⁻²s⁻¹ from cool-white fluorescent tubes. The strains with strain designation beginning with “ANT”, strain KD10 and CCMP 2086 were isolated and maintained at 6°C. The cultures were transferred every 2 weeks to fresh medium.

**Growth at different temperatures**

We assessed the growth performance of the 40 strains at 7 different temperatures (0.2, 5, 10, 15, 20, 25 and 33°C). Each treatment was replicated four times. Cells for growth experiments were harvested from exponentially growing cultures and inoculated in 24-well plates at a cell density of ~3,000 cells mL⁻¹. Light conditions and culture medium were identical to those described above for the stock cultures. We monitored growth by pulse amplitude modulated (PAM) fluorescence (MAXI Imaging PAM fluorometer, Walz, Germany). We used the minimum fluorescence yield F₀ as a proxy for biomass (Honeywill et al., 2002). We determined the growth rate during the exponential growth phase (4–5 d) as the slope of the linear regression of log₂-transformed F₀ fluorescence versus time for individual cultures. The relation between growth rate and temperature was modeled as in Rinnan et al. (2009) using the following function:

\[
\mu = a (T-T_{\text{min}})^{(1-e^{\beta(T-T_{\text{opt}})})}
\]

\( T_{\text{max}} \) and \( T_{\text{min}} \) are the maximum and minimum temperature permissible for growth. The parameter “a” is the slope and \( b \) is a fitted parameter related to the decrease in growth above \( T_{\text{opt}} \). The equation was fitted to the growth at different temperatures for each replicate using Statistica 6.0 (StatSoft, Tulsa, OK, USA). Optimal temperature and the temperatures (both low and high) at which the growth was 20% of the maximal growth rate were then calculated using a function calculator.
Chapter 3

Phylogeny

We inferred the evolutionary history of the 40 *Cylindrotheca* strains from a multi-locus DNA dataset. We selected five DNA regions, i.e. the nuclear ITS region (consisting of ITS1, 5.8S rRNA gene, and ITS2) and D1/D2 LSU rDNA, the chloroplast RUBISCO large subunit gene (*rbcL*) and *psbA* and the mitochondrial gene *coxi*. Cells for DNA extraction were harvested from exponentially growing cultures and pelleted by centrifugation. We extracted DNA using the bead-beating method with phenol extraction and ethanol precipitation as described by Zwart (1998). Sequence data were obtained using previously published PCR primers and protocols (*rbcL* and ITS: Vanelslander et al. (2009), *coxi*: Evans et al. (2007), LSU rDNA: Vanelslander et al. (submitted), *psbA*: Souffreau et al. (submitted). All sequences generated during this study were deposited in Genbank (accession numbers XXX-XXX, will be added later on).

We automatically aligned the sequences using MAFFT v6.843b (Katoh et al., 2005) and removed ambiguous positions from the ITS alignment with Gblocks 0.91b (Talavera & Castresana, 2007) allowing a maximum of 8 nonconserved contiguous positions. Data for some DNA regions were missing for several species (mainly due to erratic performance of the *coxi* primers), but the concatenated data matrix was 84.9 % filled.

We performed Bayesian phylogenetic inference (BI) on the concatenated dataset using MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). We used a GTR+I+G model in which each protein-coding gene (*coxi*, *psbA* and *rbcL*) was partitioned into three codon positions and LSU rDNA and ITS rDNA were treated as separate partitions resulting in 11 partitions. *Pseudo-nitzschia pungens* was chosen as outgroup. All parameters were unlinked between partitions. Two independent runs of four incrementally heated Metropolis-coupled Monte-Carlo Markov Chains (MCMC) were run for 20 million generations using default settings. Runs were sampled every 1,000th generation, and convergence and stationarity of the log-likelihood and parameter values was assessed using Tracer v.1.5 (Rambaut and Drummond, 2007). The first 4 million generations were discarded as burn-in and a majority rule consensus tree was generated.

We estimated divergence times under a relaxed molecular clock using an uncorrelated lognormal model in BEAST v1.4.6 following
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Souffreau et al. (submitted). We used one single calibration point, being the split of *C. closterium* and *C. fusiformis* estimated at 9 Ma (Sorhannus, 2007). The resulting chronogram displayed such a large uncertainty of divergence times that we did not include it in further analyses and it is not shown in this manuscript.

Integration of ecological data and phylogenetic information

Most analyses of trait evolution require ultrametric trees in which branch lengths are proportional with time, and not the estimated amount of molecular evolution inferred by our Bayesian analysis. Therefore, we converted our consensus phylogram into a chronogram using penalized likelihood rate smoothing (Sanderson, 2002) in the R package Ape (Paradis et al., 2004) with a lambda value of 0.1. Current traits were plotted on this chronogram to visualize trait diversity. We further visualized trait diversity by plotting traitgrams (Ackerly, 2009) which draw phylogenies in which the y position of nodes and tips corresponds to the value of a continuous trait variable, and x position corresponds to node depth using the R package Picante (Kembel et al., 2010).

We quantified the degree to which phylogenetic relatedness predicts trait similarity by calculating the K statistic presented by Blomberg et al. (2003) using the R package Picante. This statistic compares the observed phylogenetic signal in biological traits to the signal expected under a Brownian motion model of trait evolution using the same topology and branch length of the phylogeny. This Brownian motion model calculates evolution of traits as a random walk along lineages in which the change in traits at each time unit is sampled from a normal distribution with a mean of zero and a certain variance. To test for statistical significance of this K value, we performed 1,000 simulations to compare the observed signal with the distribution of randomized values. K values range between zero and infinity. K > 1 mean that traits of related lineages are more similar than expected on the basis of Brownian motion, which is often used as an indication of phylogenetic niche conservatism (Losos, 2008). K values = 1 correspond to a Brownian motion process and also suggest a certain degree of conservatism in trait evolution. K values < 1 suggest lower than expected ecological similarity between closely related lineages and may result from divergent selection.
Because the K statistic is a standardized value, it can be used to compare patterns of trait evolution in different clades.

We compared the distribution of trait disparity within and among clades by constructing disparity-through-time plots (Harmon et al., 2003) in the R package Geiger (Harmon et al., 2008). For each interior tree node, mean relative trait disparity is calculated among all subclades present at that time. This method thus provides a running average trait disparity through time and compares this to the expected trait disparity under a Brownian motion model.

The phylogenetic comparative methods used here are based on species-specific trait means and do not incorporate within-species variation. Therefore, we pruned the phylogenetic tree by removing strains with identical sequences and assigning their mean trait value to the single sequence retained. This is not ideal because it removes part of the observed trait variation, but otherwise there would be an artificial increase in the number of clades (each strain is seen as a different lineage) which would bias the results in the case of lineages with more strains.

Sea surface temperature

Temperature traits were plotted against the yearly average sea surface temperature (SST) from their sampling origin. SST values were the average SST between 1971 and 2000 and were obtained from the National Oceanic & Atmospheric Administration (NOAA, http://www.esrl.noaa.gov/psd/ accessed on March 10, 2011).

Results

Bayesian phylogenetic analysis of the concatenated alignment of rbcL, psbA, LSU rDNA, ITS and cox1 sequences (a total of 3780 characters) resulted in a well-resolved Cylindrotheca phylogeny (fig. 2). We morphologically assigned strains to C. cf clustertium and C. fusiformis. Our phylogeny clearly indicates that these morphospecies consist of a diverse set of genetic lineages, possibly representing cryptic species. There appears to be no pronounced geographic pattern in the phylogenetic tree, especially when we added rbcL and ITS sequence data of Chinese strains.
from Qingdao (Fig. 3) (Li et al., 2007), a strain from Maine (CCMP339). Lineages containing Chinese strains didn’t group together but were dispersed over the phylogeny, even though no lineages were shared between temperate Atlantic and Chinese strains.

We examined growth and survival of the *Cylindrotheca* strains across a temperature range from 0.2 to 33 °C. Strains from different climate zones strongly differed in their ability to cope with low and high temperatures (Fig. 2). Strains sampled from areas with cold climates (i.e. Spitsbergen, Baffin Bay, Antarctica) showed a limited ability to grow at temperatures >20°C, while strains from tropical and subtropical regions (Coral Sea, Gulf of Oman) had strongly reduced growth rates at temperatures <10°C. Strains from temperate regions had a broader temperature range. Yearly average sea surface temperature from the sampling location was significantly related with optimal growth temperature (Spearman $R = 0.625$, $P = 0.00001$), low temperature threshold for 20% growth ($R = 0.590$, $P = 0.00006$) and high temperature threshold for 20% growth ($R = 0.700$, $P < 0.00001$) (Fig. 4). We also observed a strong correlation between growth at high and low temperatures ($R = 0.652$, $P < 0.0001$) (Fig. 4), suggesting that species that are able to grow at low temperatures are on average less able to grow at high temperatures and vice versa.
Figure 2. Consensus tree from a Bayesian analysis of five molecular loci. Numbers at nodes are posterior probabilities indicating the support for each node; branch lengths are proportional to sequence change. For each strain the growth rate as a function of 7 different temperatures is shown.
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**Figure 3.** Consensus tree from a Bayesian analysis of five molecular loci using the sequences from fig 2, but with additional strains added (only *rbcL* and ITS data for these added strains). Strains indicated in grey were not used for the ecophysiological experiments and thermal niche analyses. The origin of strain M87326 is unknown.
Figure 4. Plots of niche traits versus average annual mean sea surface temperature (SST) A: Optimal growth temperature in function of annual mean SST. B: Lower limit for 20% growth versus mean annual SST. C: Upper limit for 20% growth versus mean annual SST. D: Upper limit for 20% growth versus Lower limit for 20% growth.
Figure 5. Consensus chronogram with the current temperature traits plotted on the tree tips.
To deduce patterns in niche evolution we first plotted the temperature trait values of the individual strains on the chronogram (Fig. 5) and made traitgrams to visualise the distribution of traits along the phylogeny (Fig. 6). Using the K estimate of Blomberg et al. (2003), we found very weak phylogenetic signals in optimal temperature ($K = 0.261$, $P = 0.0009$), low temperature thresholds for 20% growth ($K = 0.221$, $P = 0.0019$) and high temperature thresholds for 20% growth ($K = 0.353$, $P = 0.0019$).

The disparity-through-time plots (Fig. 7) confirmed the findings from the K statistic and showed that trait diversification is higher than expected under a Brownian motion model of evolution. The plots further show that there is mainly a trait diversification in low temperature thresholds and that trait disparity starts already at the origin in the clade and is almost constantly higher than expected under a Brownian motion model.

**Figure 6.** Phylogenetic traitgrams with lineages arranged along a continuous trait axis (low and high temperature threshold and temperature optimum) and connected by the underlying phylogeny.
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**Discussion**

The well-resolved, five-gene phylogeny combined with information on the fundamental temperature niche enabled us to infer thermal niche evolution within marine lineages of the diatom genus *Cylindrotheca*. We only observed a weak phylogenetic signal in thermal niche evolution, which suggests that closely related lineages tend to differ more in their
temperature niche than expected on the basis of a Brownian motion model. Phylogenetic signals can also be reduced when there is convergence in traits in distantly related lineages, like we observed for the cold adapted strains ANT105, ANT604, ANT1001 and CCMP2086. However, despite the overall low phylogenetic signal, conservatism in traits does appear to exist in some clades. For example, in the case of the three Scottish strains which originate from the same climate conditions but belong to different lineages (CCAP 101708, CCAP 101709 and CCAP 101711), we observed that the strain which has cold adapted sister lineages in the Antarctic and Spitsbergen (CCAP 101711) is unable to grow at 25°C, while the two other strains do show higher temperature thresholds.

The K statistic is only a measure of a pattern and the interpretation of the K statistic in terms of niche conservatism and adaptive radiation should be done with care (Ackerly, 2009). K values should not be light-heartedly attributed to a certain evolutionary process because very different evolutionary processes can produce similar results in terms of phylogenetic signal (Revelle et al., 2008). Low K values could be caused by a combination of adaptive evolution and frequent habitat shifts between closely related lineages. Low K values however can also result from constant stabilizing selection towards a single peak (Revelle et al., 2008) in which some lineages will adopt extreme niches, while most will be drawn back towards traits optimal for more common conditions (Ackerly, 2009). Low K values can also arise by a random walk pattern with constant evolutionary bounds such as a certain physiological constraint on the occurrence in particular conditions. Bounded evolution however should lead to a uniform distribution of trait values (Ackerly, 2009), which is not observed in our data.

The disparity-through-time plots show that there is higher trait diversification than expected under a Brownian motion model of evolution, especially for growth at low temperatures and at intermediary phylogenetic distances. It is important to recognize that our molecular phylogeny is far from complete. An extended sampling effort would certainly increase the number of extant clades we detected so far. In addition, extinct Cylindrotheca species are very hard to detect, given their very weakly silicified cell wall and thus no fossil records. In an incomplete molecular phylogeny, the internal nodes near the root will be on average more often included than nodes near the tips because deeper nodes have more descendants (Pybus & Harvey, 2000). This can lead to
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incorrect conclusions about rates of speciation and trait variation through time. So the interpretation of disparity-through-time plots should be done with caution.

Our results are similar to those of Ackerly et al. (2006), Evans et al. (2009), Knouft et al. (2006) and Graham et al. (2004) who found that climatic tolerances are evolutionary highly labile and observed strong variation in climatic tolerances between closely related lineages in a range of terrestrial organisms (the woody plant group *Caenothus*, evening primroses, *Anolis* lizards and dendrobatid frogs).

A comparison with other algae is difficult, since to our knowledge only three other studies have performed an analysis of thermal niche evolution in algae using a combination of phylogenetic information and data on niche preferences. Verbruggen et al. (2009) studied the climatic niche evolution in the seaweed genus *Halimeda* and concluded that there was a strong phylogenetic signal in the niches for four out of five sections, with most lineages displaying a conserved preference for high average temperatures and low nutrient levels. Several lineages in a fifth clade however did show several independent adaptations to colder nutrient-rich waters.

In a second study, Breeman et al. (2002) determined the evolution of thermal niches of related lineages in the green alga *Cladophora* and found differentiation in cold tolerance. Interestingly, they also found a negative relation between the capacities to grow at high and low temperatures. Such correlation can be caused by two different mechanisms. One is antagonistic pleiotropy (Angilletta et al., 2003, Zhan & McDonald, 2011) in which mutations in a single gene result in adaptation to one temperature regime but negatively affects growth at other temperatures. This would constitute a true trade-off between abilities to grow at different temperatures. Another possibility is mutation accumulation (Cooper et al., 2001) in which different sets of genes are responsible for a good performance in different environments. During adaptation in one environment, those genes that are no longer under selective pressure (e.g. those inferring heat tolerance in polar environments) become non-functional by the gradual accumulation of mutations through genetic drift. At the moment we can not distinguish between these two possibilities because we do not know the genes responsible for the adaptation to high or low temperatures.

In a third study, Souffreau et al. (unpublished) recently compared thermal adaptation of different lineages within the cosmopolitan...
freshwater diatom *Pinnularia borealis*. Although the number of sampled locations is limited, they observed a clear separation between a group of cold-adapted lineages from Chili, Antarctica, Mongolia and the French Alps/Belgium and a group of temperate climate adapted lineages from Canada/Czech Republic, Belgium and Canada/Belgium. The divergence between both groups of lineages is estimated at *ca* 22 million years ago.

Some other studies on microalgae that were initially not meant to compare niche characteristics and phylogenetic relations can nevertheless provide further information. Several studies on biogeographic distribution patterns and phylogenetic relationships in marine microalgae hint that there can be a high capacity for adaptation to new environments. Casteleyn et al. (2010) demonstrated the formation of distinct lineages within the marine planktonic diatom species *Pseudo-nitzschia pungens* during the Pleistocene, with a lineage typical for tropical to warm-temperate waters, an antitropical (temperate) lineage and a third lineage in the northeastern Pacific. Logares et al. (2008) demonstrated the rapid evolution of a set of closely related marine dinoflagellate lineages which inhabit a broad range of habitats from freshwater to marine, yet they all were typical for low temperature conditions.

Several laboratory experiments have demonstrated that microbial populations can rapidly adapt to stressful and selective environments (Vanormelingen (unpublished), Bell & Reboud, 1997, Bell & Gonzalez, 2009) and that adaptation depends on population sizes and the extent of genetic variation, next to mutation rates and the frequency of sexual reproduction and the strength of the selection pressure. Micro-algae often have extremely large population sizes, especially in marine environments, and can harbor pronounced genetic variation within species (De Bruin et al., 2004, Vanormelingen et al., 2009). In combination with a high dispersal potential in the marine environment (Cermeño & Falkowski, 2009) this could set the stage for rapid adaptation to new environments. To which extent divergent adaptation to a variety of environmental conditions is responsible for the strong diversification in the *Cylindrotheca* lineages is difficult to deduce from correlative analyses as ours. Did ecological adaptation spur speciation or vice versa? Several studies on diverse taxa (but not micro-algae) indicate that niche shifts due to natural selection trigger rapid divergence and even reproductive isolation (Losos et al., 1997, Funk, 1998, Filchak et al., 2000, Jiggins et al., 2001, Rundle & Nosil, 2005, Funk et al., 2006). On an evolutionary
time scale, this could result in the here observed repeated temperature niche shifts.

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Chapter 3


Chapter 4

Hydrodynamic Disturbance Governs Diversity and Biomass Patterns of Estuarine Benthic Diatoms

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Unpublished manuscript
Abstract

We examined the relations between hydrodynamic disturbance, biomass, species diversity and functional group turnover in estuarine intertidal microphytobenthos (MPB). To this end we analysed an extensive diatom data set representative of the variation in salinity, hydrodynamic disturbance and standing stock biomass of intertidal flats of the Westerschelde estuary (The Netherlands). Total MPB species richness displayed a unimodal relationship with hydrodynamic disturbance and standing stock biomass. In contrast, evenness increased with increasing hydrodynamic disturbance and decreasing biomass. Changes in diversity reflected a strong turnover in functional group composition along the biomass and hydrodynamic disturbance gradients, and were visible at different hierarchical levels of functional group classification. Maximal functional diversity was observed at intermediate levels of habitat disturbance.
Introduction

How species diversity and coexistence change along environmental gradients has been a long-standing question in ecology. One of the most influential hypotheses accounting for spatial variation in diversity invokes disturbance (Grime, 1973, Connell, 1978). The effect of disturbance on diversity, formalized in the Intermediate Disturbance Hypothesis (IDH, Grime, 1973, Connell, 1978), predicts that intermediate disturbance promotes maximal diversity. The basic idea behind the IDH is that exposure to low disturbance results in competitive species excluding inferior competitors resulting in low diversity. Increasing disturbance will diminish competition intensity by killing or removing cells and making space available for settling of inferior competitors resulting in the coexistence of competitive and disturbance tolerant species leading to higher species richness. With further increasing disturbance, competitive species become unable to cope with disturbance and only disturbance resistant species remain. Many empirical studies confirmed this hypothesis (Sousa, 1979, Molino & Sabatier, 2001, Svensson et al., 2007), though there remains considerable debate about the importance of competition along disturbance gradients (Viole et al., 2010) and the necessity of patchiness in disturbance events (Roxburgh et al., 2004). Reviews by Mackey & Currie (2001) and Hughes et al. (2007) revealed that in less than 25% of the studies on disturbance – species richness (SR) relations a unimodal relation was demonstrated while only 11% of studies provided evidence for such a relationship when evenness was used as a measure of diversity (Mackey & Currie, 2001). One reason why so many observations are inconsistent with the IDH is that this hypothesis relies on a number of assumptions/prerequisites, which are seldom accounted for, including the occurrence of competitive exclusion, the need of a large regional species pool, a trade-off between competitive and disturbance resistance related traits and the occurrence of multiple succession stages (Svensson et al., 2007).

In addition to disturbance, productivity (PR) - or its often used surrogate standing stock biomass (Mittelbach et al., 2001, Irigoien et al., 2004) - is another main driver of diversity patterns. Many studies have explored the shape of this relation and also often found unimodal or “hump-shaped” relations, especially in plant communities (Grime, 1973, Mittelbach et al., 2001). However, biomass can be a very poor surrogate for productivity (Whittaker, 2010). This notion is important, as SR-PR
studies focus on productivity because it can be used as a proxy for limiting resource supply rate. Ecological theories predict that species richness should be driven by the supply rate (species-energy theory, Wright, 1983) and the relative ratios of limiting resources (resource ratio theory, Tilman, 1977) that regulate the number of competing species that can locally coexist (Gross & Cardinale, 2007, Cardinale et al., 2009). Based on humped biomass diversity relations, Grime (1973) developed his classical competitive exclusion theory as an explanation for these unimodal relationships. He stated that maximal diversity at intermediate biomass values was due to the coexistence of species belonging to different plant strategies. Communities with high biomass are species poor because they are dominated by competitive species and low biomass communities are again species poor because they lack the competitive species.

Mittelbach (2001) pointed to the fact that our knowledge of species richness - biomass and productivity relationships is mainly based on particular taxonomic groups (vascular plants, mammals, aquatic invertebrates) and therefore may be biased. Since 2001, several studies investigating the SR-PR relation focused on other taxonomic groups, but aquatic microbial communities for instance are still underrepresented (but see Li, 2002, Horner-Devine et al., 2003, Irigoien et al., 2004, Cardinale et al., 2006).

In this paper, we examine the relationships between the hydrodynamic disturbance, biomass and species diversity in estuarine intertidal microphytobenthos (MPB). MPB plays an important role in the ecology of estuarine systems. They provide up to 50% of the primary production (Underwood & Kromkamp, 1999), contribute to sediment stabilization through the production of extracellular polymer secretions (Decho, 2000) and influence nutrient fluxes between sediment and water (Sundbäck et al., 2000). In our study area, the Westerschelde estuary, MPB is largely made up of benthic diatoms.

Estuarine MPB communities are subject to strong species sorting effects due to the structuring of communities by salinity, light exposure and hydrodynamic forces (Admiraal, 1984, Sabbe & Vyverman, 1991, Underwood, 1994, Paterson & Hagerthey, 2001). Dispersal is high due to erosion/sedimentation dynamics and by import of propagules from upstream and from the sea. There is a large gradient in salinity spanning more than 60 km. A second important gradient is the degree of hydrodynamic disturbance. Intertidal areas experience strong wave action
that carries away fine sediment particles and leave coarser, well sorted sand, while finer sediment like silt and clay particles are deposited in more sheltered areas (Oh & Koh, 1995). The degree in wave action thus produces a continuous gradient in sediment grain size which strongly influences other environmental factors such as water content (Paterson et al., 2000) and nutrient concentrations (Paterson & Hagerthey, 2001). At the microscale, there are steep gradients in nutrients, light and oxygen (Admiraal, 1984, Paterson & Hagerthey, 2001). All these gradients result in a very heterogeneous intertidal environment at various spatial and temporal scales. This heterogeneity creates various different niches for estuarine benthic diatoms. Benthic diatom diversity in estuaries is therefore high, not only from a taxonomic point of view (> 200 species commonly present in the Westerschelde estuary) but also from a functional point of view. Benthic diatoms can be classified in three large functional groups, namely the motile epipelic diatoms, the sand grain-attached epipsammon and tychoplankton (Admiraal, 1984).

In the past, species diversity and distribution patterns have mainly been assessed for epipelic assemblages (Underwood, 1994). We explored this relationship using a large dataset comprising monthly sampling of 32 stations in the Westerschelde estuary during one year (>360 samples) covering a wide range of hydrodynamic disturbance, salinity and MPB biomass. Our main questions were: 1) what is the relationship between hydrodynamic disturbance and diatom species richness and evenness; 2) what is the relation between diatom diversity and diatom biomass; and 3) how do the different functional groups respond towards disturbance and biomass gradients?

Material and Methods

Study site

The Westerschelde is the meso- to euhaline part of the Schelde estuary (Belgium/The Netherlands). It is a macrotidal coastal plain estuary subject to considerable organic pollution and eutrophication. The Westerschelde is well mixed and characterized by a complex morphology with tidal channels surrounding several large intertidal flats and salt marshes. Residence time of the water in the Westerschelde estuary ranges
from one to three months (depending on the season) resulting in a rather
stable and gradual salinity gradient (ranging from 3 to 32 psu in the
Westerschelde) that is primarily determined by the magnitude of the
river discharge and to a lesser extent to the tidal oscillation, which is of
smaller amplitude (Meire et al., 2005).

Sampling campaign

Thirty-two sampling stations (Fig. 1) were sampled monthly during one
selected in order to cover the entire range in salinity and sediment types
in the Westerschelde estuary. Sediment cores (diameter 22 mm) from the
upper 1 cm of the sediment were taken at low tide and fixed with 4%
formalin. Five cores from each locality (within 1 m²) were combined to
compensate for microscale differences in diatom composition and
abundance.

Figure 1. Map of the Westerschelde estuary showing the position of the salinity zones
(McLusky, 1993) and the location of the sampling stations. Intertidal areas below
mean high water are shown in light grey, intertidal and supra-tidal areas above mean
high water (predominantly saltmarshes) in dark grey. Az: Appelzak, Bn: Baalhoek,
Hp: Hooge platen, Ks: Konijnenschor, Mp: Molenplaat, Nb: Nauw van Bath, Ph:
Platen van Huls, Ps: Paulinaschor, Ra: Rammekenshoek, Sn: Staartse Nol, Te:
Terneuzen.
Species composition and diversity

Part of the homogenized, combined samples was oven-dried at 60 °C. One gram sediment dry weight (SDW) of each sample was then oxidized with hydrogen peroxide (27%) and acetic acid (99%) (Sabbe, 1993). The sediment was then washed 3 to 4 times with distilled water and diluted to a final volume of 50 ml. After thorough homogenization a subsample of 50 µl was taken with a micropipette, transferred to a coverslip and air-dried. Permanent preparations were made with Naphrax mounting medium. On each coverslip all diatom valves in a transect of known surface area were identified and counted. Allochtonous species (planktonic and freshwater) were omitted from the analyses. Cell counts of 400 cells per sample can not adequately estimate the occurrence of rare species (Alverson et al., 2003), therefore we also omitted species which occurred less than three times in the dataset. The original dataset contained 294 taxa and after omission of these species it contained 193 species. The counted surface was then extrapolated to the whole surface of the dried drop, thus allowing us to determine the total number of diatoms in one gram SDW. For each slide a minimum area of 5000 by 100 µm was counted. Counts were continued beyond this minimum area until at least 400 valves were counted. In some samples, diatom densities were very low; a maximum of ten transects of 5000 by 100 µm was then scanned for diatoms.

We assigned species to functional groups based on the following traits: cell-size and shape, hold-fast mechanisms and motility. These traits are all valuable for characterizing functional traits in benthic microalgae. Cell size determines specific physiological activities such as growth (Weithoff, 2003). Furthermore, cell-size and shape jointly determine the surface to volume ratio which in turn influences nutrient uptake. Hold-fast mechanisms evidently determine to which extent cells are able to cope with hydrodynamic disturbance. Motility is undeniably an important trait in intertidal mudflats as these environments are characterized by steep gradients in light and nutrients and enables cells to position themselves in favorable patches. Inhibiting and limiting light conditions are often only a few hundred µm away from each other. Moreover, nutrients such as silica can become limiting in the upper part of the sediment when production is high (Jesus et al., 2009). It has recently been shown that epipelic diatoms display the ability to sense
silica concentration gradients and migrate to favourable patches (Gillard et al. unpublished).

Based on these groups we classified benthic diatom species into the motile epipelic diatoms, the sand grain-attached epipsammon and tychoplankton, functional groups which are well established in microphytobenthos research (Paterson & Hagerthey, 2001). Within the epipsammon, we further distinguished between adnate, stalked and small (<10µm) motile species (Hamels et al., 1998). The epipelon was divided into small-celled (10-20µm) and big-celled (>20µm) diatoms (fig. 2) will explore if these autecological traits are able to predict the effect of disturbance on functional group species richness.

Cell biovolumes of each taxon was calculated using linear dimensions (length, width and height) and a standardized set of equations for biovolume calculations for the different diatom genera (Hillebrand et al., 1999). The total biovolume per sample was calculated based on the cell densities and the mean biovolume for each diatom species.

While the oxidation method ensures that all diatom cells are quantitatively separated from the sediment and also allows accurate identification, it does not allow to distinguish between dead and living cells, which could lead to an overestimation of the total diatom biomass. We therefore estimated the relative proportion of living cells in the different functional groups in about 30 fixed samples stained with Bengal Rose, which allowed us to distinguish between empty frustules and those with a cell content (the latter were referred to as living diatoms) (Hamels et al., 1998). These countings demonstrated that the oxidized counts do supply realistic estimations of the absolute biomass values since the ratios of the living to dead cells of the epipelon closely follow trends in cell abundance (Hamels et al., 1998). On average, the living:dead ratio was relatively stable, between 40 to 60% of the cells was alive. Using these living/dead ratios, the original quantitative counts on the permanent preparations were then corrected to yield the total number of living diatoms per gram SDW.
Figure 2. The different functional groups of diatoms that occurs in the Scheldt estuary. The epipsammon (comprising the stalked, adnate and motile <10μm groups), which is the smallest groups, can grow attached to individual sand grains. The epipelon (comprising the motile 10-20 μm and the > 20 μm groups) consists of larger, motile diatoms that form dense biofilms. The tychoplankton has a more amphibious life style.

Granulometric analysis

Granulometric analyses of the sediment (top 1 cm) were performed every two months at each station; for the intervening sampling months, values were obtained by linear extrapolation. Size analysis was performed with a Coulter Counter (LS100, Coulter Electronics, UK). By adopting a grade scale (the Wentworth scale, Buchanan & Kain, 1971), we then assessed what sediment volume percentage was present in each size class. The following particle size classes were distinguished: clay (< 3.9 μm), silt (3.9 -63 μm), very fine sand (63-125 μm), fine sand (125-250 μm), medium sand (250 μm-500 μm) and coarse sand (500 μm- 1000 μm).
It is well known that hydrodynamic disturbance strongly determines the sediment composition of intertidal sediments (Oh & Koh, 1995). We therefore conducted a principal component analysis (PCA) based on the standardized abundances of the particle size classes and median grain size and used the first principal component axis sample scores as a proxy for hydrodynamic disturbance of the sediment (Van Colen et al., 2010). The first axis explained 64.1% of the variation in granulometry and was strongly correlated with the silt fraction ($r=0.963$, $p<0.001$).

**Statistical analysis**

We calculated both species richness and Pielou's evenness index (based on cell abundance) for each of the 360 samples. Trends in total species richness, functional group species richness and total evenness along log-transformed biomass and hydrodynamic disturbance were explored using linear and quadratic regressions for which we checked the homogeneity and homoscedasticity. We used the Akaike information criterion (AIC) to select the most reliable model. If the quadratic regression was selected, we used the Mitchell-Olds and Shaw (MOS) test (Mitchell-Olds & Shaw, 1987) to determine whether there was a significantly unimodal (or so called 'humped') shape and to estimate the peak of the unimodal curve. We further verified if the estimated peak diversity is significantly greater than the diversity at the minimum biomass/disturbance and less than the diversity at the observed maximum biomass/disturbance, i.e. if the maximum richness is reached within the observed range of biomass/disturbance. The MOS test is a classical method to look for hump-shaped species richness patterns (Mittelbach et al., 2001, Chase & Leibold, 2002) and was conducted using the VEGAN package in R (Oksanen et al., 2008).

Salinity gradients in estuaries strongly affect diatom species distributions and richness (Sabbe & Vyverman, 1991). As our sampling campaign covered a broad range of salinities (between 8.5 and 35 psu for the interstitial water), we tested for the effect of salinity on species richness. As we found a weak but significant trend in species richness along the salinity gradient (supplementary table 3, total species richness \( = 41.30 + 0.22 \times \text{salinity} \), \( F = 11.44, \quad P = 0.0007, \quad R^2 = 0.028 \)), we recalculated the above regressions between diversity and biomass/disturbance, but now using the residual species richness after accounting for the salinity trend (Cardinale et al., 2006).
Beta diversity was quantified using the Bray-Curtis dissimilarity index (based on standardized quantitative abundance data) and the Jaccard’s index (based on presence/absence data).

We compared the diversity in samples from different silt content classes (0-1%, 1-5%, 5-10%, 10-20%, 20-40%, >40% silt) using Analysis Of Similarity (ANOSIM, (Clarke, 1993), implemented in the software package PRIMER 6 (Clarke & Gorley, 2006) based on the Bray-Curtis and on the Jaccard index. ANOSIM produces a test statistic R which lies between 0 and 1 depending on the similarities between groups. An R value of 1 indicates maximal dissimilarity, while 0 stands for full similarity. Contrasting quantitative and qualitative metrics enables one to assess whether dissimilarities between groups are mainly due to compositional change or change in relative abundances (Schaefer et al., 2005).

Results

Diversity along a disturbance gradient

Total diatom species richness showed a significant quadratic relation with hydrodynamic disturbance (Table 1, Fig. 3A) and the MOS test indicates that the relation is hump-shaped. Samples from stations associated with low and high disturbance displayed low diversity, while samples from intermediately disturbed stations had the highest diversity. The Pielou’s evenness index (Fig. 5A) showed a significant negative correlation with increasing hydrodynamic disturbance (Spearman Rank R = -0.636, P = 0.0001).

The species richness of the epipsammon showed a significant linearly but not unimodally decreasing relation with decreasing hydrodynamic disturbance (Table 1, Fig. 3A). The species richness of the epipelic and tychoplanktonic functional groups displayed a significant quadratic relation with hydrodynamic disturbance (Table 1, Fig. 3A). The MOS test showed that both relations were unimodal and that the estimated peak richness of both groups was located within the observed range of disturbance values and is expected at 0.419 disturbance and
1.559 (PCA axis 1 values) disturbance for the epipelon and tychoplankton respectively.

Species richness of the adnate epipsammic diatoms linearly decreased with decreasing hydrodynamic disturbance (Table 1, Fig. 3C). The small motile (<10 μm) epipsammic diatoms showed a quadratic relation with hydrodynamic disturbance (Table 1, Fig. 3C). The MOS test indicated that the relation was unimodal, but the estimated peak diversity was not significantly greater than the diversity at the maximal hydrodynamic disturbance. The stalked epipsammic diatoms showed a quadratic (Table 1, Fig. 3A), unimodal (MOS test) relation with disturbance with estimated peak diversity at -1.075. The epipelic diatoms between 10 and 20 μm significantly decreased along decreasing hydrodynamic disturbance (Table 1, Fig. 3A). For the epipelic diatoms > 20 μm species richness, a linear regression was not possible as the assumption of homoscedasticity was not fulfilled. Therefore we used a non parametric Spearman rank correlation and found a positive correlation between decreasing disturbance and species richness of motile diatoms > 20 μm (R=0.2373; p=0.000005).
Figure 3. Species richness (SR) along disturbance and total biomass gradients. (A-B): Total SR, tychoplanktonic, epipsammic and epipelic SR along the disturbance (A) and biomass (B) gradient (lowest disturbance value = maximal disturbance). Only significant regressions are plotted. Upper black line: regression for total SR, dashed black line: epipsammic SR, grey line: tychoplankton SR, lower black line: epipelic SR. (C-D): SR of 3 epipsammic growth morphologies (adnate, < 10μm motile and stalked) along disturbance (C) and biomass (D) gradient. Black line: regression for motile < 10 μm SR, grey line: adnate SR, dashed black line: stalked diatom SR. (E-F): SR of 2 epipelic growth morphologies (motile 10-20 μm and motile > 20μm) along the disturbance (E) and biomass (F) gradient. Regression equations are given in Tables 1 and 2.
Table 1. Regression equations for total and functional group species richness vs. hydrodynamic disturbance (linear: $Y=a+bX$ or Quadratic: $Y=a+bX+cX^2$). Regressions are shown in Fig. 3. Significance codes: 'N.S.' $P > 0.05$ ; '*' $0.01 < P < 0.05$ ; '**' $0.01 < P < 0.001$ ; '***': $P < 0.001$. AIC: Akaike's information Criterion. N.A. not applicable. SE: Standard Error. The AIC value for the most reliable model is shown in bold.

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Diversity – disturbance in benthic diatoms

**Table 2.** The regression equations for the total species richness and functional group species richness vs. total standing biovolume stock (linear: \( Y = a + bX \) or Quadratic: \( Y = a + bX + cX^2 \)). Regressions are shown in Fig. 3. Significance codes: 'N.S.' \( P > 0.05 \); **' 0.01 < \( P < 0.05 \); ***' 0.01 < \( P < 0.001 \); '***' \( P < 0.001 \). AIC: Akaike's information Criterion. N.A. not applicable. SE: Standard Error. The AIC value for the most reliable model is shown in bold.
The hydrodynamic disturbance is also strongly related to total diatom biomass (biomass = 0.498*disturbance + 4.755, R²=0.654, P = 2.2 \times 10^{-16}) (Fig. 4). The strong collinearity between biomass and disturbance makes it impossible to discern the relative importance of both on species richness using a multiple regression.

**Figure 4.** Relation between biomass and hydrodynamic disturbance. (Lowest disturbance value = maximal disturbance)

### Diversity along a biomass gradient

Total diatom species richness showed a significant quadratic relation with total cell biovolume (used as a proxy for biomass) (Table 2, Fig. 3B). The MOS test revealed that the relation is unimodal and hump-shaped with the estimated peak diversity within the observed biomass ranges (estimated at 23.70 \times 10^{12} \text{µm}^3 \text{biovolume/m}^2). Pielou's evenness (Fig. 5B) showed a negative correlation with increasing biomass (Spearman Rank R = -0.542, P = 0.00000).

**Figure 5.** Pielou's evenness along the disturbance and biomass gradient.
Diversity - disturbance in benthic diatoms

The species richness of the epipsammic and epipelic functional groups also displayed a significant unimodal, humped-shaped relation with biomass (Table 2, Fig. 3B). The estimated peak epipelic diversity (using the MOS test) was located within the observed range of biomass values and is expected at 59.53 \( \times 10^{12} \) \( \mu m^3 \) biovolume/m\(^2\). The estimated peak epipsammic diversity was also within the observed range of biomass values, but it was not significantly greater than the epipsammic diversity at the minimal observed biomass. The species richness of the tychoplankton showed a significant linear positive relation with increasing biomass. Thus the different functional groups show peak diversity at distinct biomass values.

The species richness of the three different epipsammic growth forms all had a significant unimodal, humped-shaped relation with biomass (Table 2, Fig. 3D). The estimated peak adnate diversity was located at lower than observed biomass values \((0.51. \times 10^{12} \mu m^3 \text{ biovolume/m}^2)\), while the small motile and stalked diatoms showed peak diversities at 5.69 \( \times 10^{12} \mu m^3 \text{ biovolume/m}^2 \), 14.21 \( \times 10^{12} \mu m^3 \text{ biovolume/m}^2 \) and 23.4 \( \times 10^{12} \mu m^3 \text{ biovolume/m}^2 \), respectively. The epipelic diatoms between 10 and 20\( \mu m \) showed a unimodal, humped-shaped relation with biomass (Table 2, Fig. 3F). The estimated peak diversity was estimated at 2.97 \( \times 10^{12} \mu m^3 \text{ biovolume/m}^2 \) (no confidence interval could be determined). For the epipelic diatoms > 20\( \mu m \) species richness, a linear regression was again not possible as the assumption of homoscedasticity was not fulfilled. We performed a non parametric Spearman rank correlation and found a positive correlation between increasing biomass and species richness of motile diatoms > 20 \( \mu m \) (Spearman R 0.1815 p=0.00053). So again the different growth morphologies show peak diversity at different biomass values.

We repeated the above described regressions for disturbance and biomass using the residual species richness after accounting for the salinity trend and found largely the same results (supplementary table 1 and 2).

**Species turnover**

Analysis of Similarity (ANOSIM) based on quantitative and qualitative species data was used to assess differences in species composition and
species turnover along the disturbance gradient (Tables 3 and 4). As silt content of sediment samples is strongly related to the hydrodynamic disturbance proxy used during this study ($\text{log}silt = 0.534 \times \text{disturbance} + 1.02, R^2 = 0.912, P = 2.2 \times 10^{-12}$), we classified the sampling stations in different silt classes and then analyzed their similarity. The most disturbed sampling stations with a silt content between 0 and 1% had a very specific species composition which showed relatively low similarity with the 1-5% silt class and almost no similarity with the least disturbed sites (20% silt or more) (Table 3 and 4). The sampling stations with silt content $\geq 10\%$ showed high similarity in species composition. Contrasting the quantitative (ANOSIM based on the Bray-Curtis index, Table 4) and qualitative (ANOSIM based on the Jaccard index, Table 3) metrics showed that both compositional change and change in relative abundances occurred. For instance, the sampling stations with 1-5% silt content and with a 10-20% silt showed a R value of 0.39 based on qualitative data, but a R value of 0.598 based on quantitative data, showing the importance of changes in relative abundances. Overall, the strongest turnover in species occurred at low silt content (0-10% silt), but species turnover continued also at higher silt content.

Table 3. One-way analysis of similarities (ANOSIM) using the Jaccard dissimilarity index based on presence/absence data of species along different silt content percentage classes. Values given are the R statistic and the probability level (P).

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<td>1-5% silt</td>
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<td>0.008 (0.418)</td>
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<td>10-20% silt</td>
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<td>&gt;40% silt</td>
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<td>0.278 (0.003)</td>
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Table 4. One-way analysis of similarities (ANOSIM) using the Bray-Curtis index based on standardized species abundance data along different silt content percentage classes. Values given are the R statistic and the probability level (P).

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Diversity – disturbance in benthic diatoms

**Diatom cell size**

Differences in species richness, functional groups and species composition along the biomass and disturbance gradients coincided with changes in average cell size and cell size distribution within samples. There was a significant increase in average cell size with increasing biomass values (Spearman Rank $R = 0.502$, $P = 0.0000$; Fig. 6B) and a significant increase in average cell size with decreasing hydrodynamic disturbance (Spearman Rank $R = 0.496$, $P = 0.0000$, Fig. 6A).

The most disturbed sampling sites with silt content between 0 and 1% were dominated by very small diatoms (<100 μm³ cell biovolume) (Fig. 7). Sampling sites with 1 – 5% silt also had a significant portion of the smallest diatoms (<100μm³), but also a second group of diatoms with cell biovolumes between 100 and 240μm³. A further increase in silt content and thus lower disturbance resulted in a gradual decrease of the <100μm³.

**Figure 6.** A: Average cell biovolume versus total biomass. B: Average cell biovolume versus disturbance (lowest value = maximal disturbance)
**Discussion**

Species richness in intertidal microphytobenthic diatom communities of the Westerschelde estuary shows a significant hump-shaped relation with disturbance (Table 2), which is in agreement with the intermediate disturbance hypothesis (IDH, Grime, 1973, Connell, 1978). As most studies to date have been focused on epipelic communities of the less disturbed, silt rich intertidal sediments, it was not known whether intertidal microphytobenthos would respond to the IDH, although (Paterson & Hagerthey, 2001) hypothesized that it would. The IDH has previously been applied to phytoplanktonic communities, in both experimental and field investigations (Reynolds et al., 1993, Sommer, 1995, Floder & Sommer, 1999), most of which also comply with the IDH.

The IDH assumes that there is a trade-off in traits that determine competitive ability and disturbance tolerance (Haddad et al., 2008), resulting in maximal diversity where competitive and disturbance tolerant species co-occur (Haddad et al., 2008). Here we assigned species
to functional groups based on the following traits: cell-size and shape, hold-fast mechanisms and motility. These functional traits were able to explain the effect of disturbance on functional group species richness. High energy conditions with strong wave action and tidal currents create strong selection gradients allowing only a small number of erosion-resistant species with hold-fast mechanisms to adapt to these conditions. Highly disturbed, coarse sediments were therefore characterized by the occurrence of mainly epipsammic diatom species and a relatively high evenness. Within the epipsammic communities we observed a turnover from adnate and small (<10µm) motile species towards stalked species along decreasing disturbance gradients.

The presence of the small (<10 µm) motile forms in the most dynamic sediments may appear contradictory at first, as it would increase the risk of resuspension. However, their motility might actually be a good adaptation to life on these larger sand grains as it enables them to go for shelter (in crevices) when circumstances are most dynamic (e.g. at high tide), while they can move across the sand grains to optimize e.g. light exposure when circumstances are less dynamic (low tide). Stalked forms were more dominant in less dynamic sediments which was also noted by (Sundbäck, 1983), as they are more prone to abrasion when the sediments become too dynamic.

With decreasing disturbance, sediments are often poorly sorted (fig. 8), containing both silt and sand fractions. Epipelic diatoms are able to colonize these sediments, mainly small motile (10-20 µm) species, while larger motile (>20 µm) taxa have higher species richness at lower disturbance. The least disturbed, silt rich, nutrient rich sediments harbour few species which can reach high dominance resulting in low evenness, suggesting strong competitive interactions. These sediments harboured mainly bigger celled epipelic (>20 µm) and tychoplanktonic species.

The turnover in functional groups coincides with strong species turnover along the disturbance gradient with a complete disparity between the least and most disturbed communities. Species turnover was especially strong at the high disturbance end. The functional and taxonomical turnover also results in a changing diatom cell size distribution along the disturbance gradient. The most disturbed sediments are dominated by very small diatoms (<100µm³), while mixed sand and muddy stations harbour several size classes and average cell size increasing with decreasing disturbance. A greater variety in cell sizes in
less disturbed sediments was also observed by Jesus et al. (2009) who hypothesized that muddier stations may provide more potential niche spaces promoting greater size variation.

Irigoin et al. (2004) and Li (2002) also observed an increasing cell size with increasing biomass in phytoplankton communities. They attributed this to size-governed protection against zooplankton predation. Here we do not have data on grazing intensity, so it is difficult to draw any conclusions about this. The occurrence of large species in the most competitive communities seemingly contradicts with their lower surface area to volume ratio causing them to have a less efficient nutrient acquisition compared to small species (Raven, 1998). However, analyses by Grover (1989) on microalgae revealed that for spherical cells, smaller cells are indeed better competitors, but large cells with an elongated shape are often better competitors than elongated small ones. Another potential mechanism is that larger cells have a better nutrient storage ability which is important in fluctuating nutrient environments (Grover, 1991, Litchman et al., 2009) such as intertidal mudflats which typically have strong nutrient concentration gradients.

![Figure 8. Sediment grain size classes against the disturbance gradient. The disturbance gradient largely coincides with a gradient in sediment composition. The most disturbed sites (negative values) contain mainly medium sized sand, while the least disturbed sites are dominated by silt and clay particles. At intermediate disturbance, fine sand (125-250µm) is the most important sediment type.](image-url)
The classical explanation of the IDH relies heavily on competitive exclusion and environmental filtering based on species-specific tolerances towards disturbance. Michalet et al. (2006) recently suggested to include facilitation into models explaining the IDH. They argued that facilitation can promote diversity at medium to high disturbance intensities by expanding the realized niche of disturbance-intolerant competitive species towards stressful conditions. Typical examples for intertidal communities are the large macroalgal canopy-forming species that protect understory algae against physical stressors (Molina-Montenegro et al., 2005). Though on another scale, facilitation through alleviation of stressful conditions could apply to microphytobenthos as well. Laboratory experiments demonstrated that single cells of epipelic diatom species have limited resistance towards resuspension by water currents, which is in contrast to epipsammic species (Harper 1977). However, natural epipelic diatom assemblages exude copious amounts of extracellular polymeric secretions (EPS), thereby creating sticky biofilms that have pronounced influence on erosion thresholds of sediments (Paterson, 1989). Thus intertidal diatom biofilms can trap and retain sediments at current velocities that would normally lead to sediment and MFB resuspension (Paterson & Hagerthey, 2001, Van De Koppel et al., 2001). Yet, different epipelic diatom species seem to have varying abilities to produce EPS and stabilize the sediment (Holland et al., 1974, Underwood, 1994, Paterson & Hagerthey, 2001). Field observations and culture experiments show that the abundant diatoms species Navicula flanatica and Cylindrothece closterium presumably have little contribution to sediment stabilization (Holland et al., 1974, Underwood, 1994, Paterson & Hagerthey, 2001) but likely take advantage of this habitat amelioration.

Next to modifying hydrodynamic disturbance, benthic microalgae can also facilitate other benthic microalgae through the alleviation of limiting conditions. Benthic diatoms often face light limitation due to wave induced burial or by self-shading in thick diatom biofilms. Some MPB species are mixotrophic and have the ability to take up dissolved organic substrates as a carbon or nitrogen source (Linares & Sundback 2006). We recently showed that a mixotrophic strain of Cylindrothece closterium was able to enhance its growth by recycling organic exudates of other benthic diatoms and was therefore facilitated in its growth by naturally co-occurring diatom species (Vanselslander et al., 2009). To which extent these facilitative interactions influence the diversity of natural communities is difficult to deduce from our field data and thus
field and lab experiments are needed to determine if facilitation actually promotes diversity in intermediate to highly disturbed sediments.

In addition to the disturbance gradient, we also assessed diversity patterns along biomass gradients. Generally the same patterns show up, which is not surprising given that disturbance and biomass are strongly correlated. Biomass is often used as proxy for productivity to study productivity-diversity relationships. This might be reasonable in some cases, but quite often there is no correlation between biomass and productivity, as shown for intertidal benthic diatoms (Forster et al., 2006). This improper use of productivity surrogates lead to the misconception that productivity-diversity relations were most frequently hump shaped (Mittelbach et al., 2001). So caution is required when choosing a measure for productivity (and thus the availability of resources that limit production).

In summary, we show that diversity of intertidal benthic diatom communities is maximal at intermediate hydrodynamic disturbance. We further show a decreasing evenness towards the least disturbed sites, indicative for strong competitive interactions. The peak in species richness could be explained by a turnover in functional groups which overlapped and coexisted at intermediate disturbance. This turnover was visible at different hierarchical levels of functional group classification and was also evident in cell size distributions along the disturbance gradient.

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Supplementary material

Table 1. The regression equations for the species richness/functional group richness vs. hydrodynamic disturbance (linear: \( Y = a + bX \) or Quadratic: \( Y = a + bX + cX^2 \)) using the residual species richness after accounting for the salinity trend. No regression given for the motile 10-20 μm and tychoplankton groups since there is no significant relation between diversity and salinity. Significance codes: 'N.S.' \( P > 0.05 \); '••' \( 0.01 < P < 0.05 \); '***' \( 0.01 < P < 0.001 \); '****' \( P \leq 0.001 \). AIC: Akaike's information Criterion. N.A. not applicable. SE: Standard Error. The AIC value for the most reliable model is shown in bold.
Table 2. The regression equations for the species richness/functional group richness vs. total standing biovolume stock (linear: $Y = a + bX$ or Quadratic: $Y = a + bX + cX^2$) using the residual species richness after accounting for the salinity trend. No regression given for the motile 10-20 μm and tychoplankton groups since there is no significant relation between diversity and salinity. Significance codes: 'N.S.' $P > 0.05$ ; **: $0.01 < P < 0.05$ ; ***: $0.01 < P < 0.001$ ; '***': $P < 0.001$. AIC: Akaike's Information Criterion. N.A. not applicable. SE: Standard Error. The AIC value for the most reliable model is shown in bold.
Table 3. The regression equations for the species richness/functional group species richness vs. salinity ($Y=a+bX$). Significance codes: 'N.S.' $P > 0.05$ ; '•' $0.01 < P < 0.05$ ; '***' $0.01 < P < 0.001$ ; '***' $P < 0.001$. AIC: Akaike's information Criterion. N.A. not applicable. SE: Standard Error.

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<th>$b \pm SE$ (p-value)</th>
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<th>$P$</th>
<th>Adj. $R^2$</th>
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<td>Total</td>
<td>41.30 ± 1.62 ***</td>
<td>0.22 ± 0.67***</td>
<td>0.00079</td>
<td>0.0282</td>
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<tr>
<td>Epipelon</td>
<td>14.79 ± 0.97 ***</td>
<td>-0.13 ± 0.04**</td>
<td>0.00113</td>
<td>0.0265</td>
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<tr>
<td>Epipsammon</td>
<td>13.92 ± 1.45 ***</td>
<td>0.30 ± 0.06 ***</td>
<td>5.681 . 10$^2$</td>
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<td>Tychoplankton</td>
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<td>0.05 ± 0.04 (N.S.)</td>
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<td>Motile &gt;20 µm</td>
<td>9.75 ± 0.70 ***</td>
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<td>Motile 10-20 µm</td>
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<td>Adnate</td>
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<td>Stalked</td>
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<td>0.06 ± 0.01 ***</td>
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</tbody>
</table>
Chapter 5

Complementarity Effects Drive Positive Diversity Effects on Biomass Production in Experimental Benthic Diatom Biofilms

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Abstract

Positive effects of species diversity on ecosystem functioning have often been demonstrated in ‘macrobial’ communities. This relation and the responsible mechanisms are far less clear for microbial communities. Most experimental studies on microorganisms have used randomly assembled communities that do not resemble natural communities. It is therefore difficult to predict the consequences of realistic, non-random diversity loss.

In this study, we used naturally co-occurring diatom species from intertidal mudflats to assemble communities with realistically decreasing diversity and analysed the effect of non-random species loss on biomass production. Our results demonstrate a highly positive biodiversity effect on production, with mixtures outperforming the most productive component species in more than half of the combinations. These strong positive diversity effects could largely be attributed to positive complementarity effects (including both niche complementarity and facilitation), despite the occurrence of negative selection effects which partly counteracted the positive complementarity effects at higher diversities.

Facilitative interactions were, at least in part, responsible for the higher biomass production. For one of the species, *Cylindrotheca closterium*, we show its ability to significantly increase its biomass production in response to substances leaked into the culture medium by other diatom species. In these conditions, the species drastically reduced its pigment concentration, which is typical for mixotrophic growth.

We show that both species richness and identity have strong effects on the biomass production of benthic diatom biofilms and that transgressive overyielding is common in these communities. In addition, we show mechanistic evidence for facilitation which is partly responsible for enhanced production. Understanding the mechanisms by which diversity enhances the performance of ecosystems is crucial for predicting the consequences of species loss for ecosystem functioning.
Introduction

The effects of biodiversity and changes in community composition on the functioning of ecosystems have been a central topic in ecological research during the past decade. The main research question has been whether ecosystems with decreasing species numbers and altered species compositions are able to maintain their functional properties and process rates. The majority of these studies demonstrate that a decline in species richness and functional diversity can adversely affect central ecosystem processes such as productivity (Hooper et al. 2005; Srivastava & Vellend 2005; Cardinale et al. 2006).

Theory suggests that positive relations between biodiversity and ecosystem functioning arise through three primary mechanisms: sampling or selection probability effects, niche complementarity and facilitation. The sampling effect refers to the increased probability of selecting species with a specific property in randomly assembled experimental treatments with a higher number of species (Huston 1997). Niche complementarity occurs when species differ in their resource requirements, resulting in lower competition from interspecific neighbours than from conspecifics. This may lead to a more complete resource use by more speciose communities (Fridley 2001). Facilitation occurs when a species modifies the environment in a way that benefits a co-occurring species (Vandermeer 1989). Niche complementarity and facilitation are collectively referred to as 'complementarity', as it is often unclear which mechanism prevails.

Although niche complementarity and facilitation are often considered to enhance ecosystem functioning with increased species richness, direct functional and ecophysiological evidence is scarce. Complementarity is generally demonstrated indirectly by comparing mixture yields with the expected yield based on the monocultures of the component species (Loreau 1998; Loreau & Hector 2001). While there are numerous examples of niche complementarity (e.g. complementary N uptake strategies in plants; Jumpponen et al. 2002), there is little direct evidence showing how it can lead to increased resource use and thus serve as a functional mechanism in positive biodiversity ecosystem functioning (BEF) relationships (Kahmen et al. 2006). Mechanistic evidence for facilitation has been demonstrated for N-fixing legumes (Temperton et al. 2007) and larvae of suspension-feeding Trichoptera (Cardinale et al. 2002), but further direct evidence for facilitation is lacking.
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The majority of the studies linking community diversity with ecosystem functioning have focused on terrestrial plant communities, whereas microbial communities have received less attention, especially in marine systems (Petchey et al. 2002; Giller et al. 2004). Microbial systems seem extremely attractive for testing BEF hypotheses because they provide high levels of experimental control and replication, and the possibility to run experiments over many generations. However, microbial BEF experiments that are relevant to natural situations are difficult to realize due to the fact that the majority of microorganisms resist cultivation in the laboratory (up to 99% of prokaryotes, Kaeberlein et al. 2002). As a result, most BEF experiments with microbial communities used artificial communities of easily cultivable taxa, which usually bear little resemblance to natural assemblages in terms of both species composition and species richness. In addition, these artificial communities are generally randomly assembled, while natural species loss is typically non-random due to differences in population size, immigration rate and specific resistance to stressors (Giller et al. 2004; Srivastava & Vellend 2005). This raises concerns about the relevance of previously observed diversity effects for natural microbial communities and renders it more difficult to predict the consequences of realistic diversity loss. The few studies that have used non-random diversity changes (Solan et al. 2004; Zavaleta & Hulvey 2004; Bracken et al. 2008) found more rapid and disproportionate loss of function than expected from randomized loss experiments.

We focused on estuarine benthic diatom (Bacillariophyta) communities which provide a useful microbial system to address the link between biodiversity and ecosystem functioning because these communities are relatively species-poor (local diversity mostly ranging from two to nine species, Forster et al. 2006). In addition, many species are cultivable (Mann & Chepurnov 2004) and readily identifiable using light microscopy (Forster et al. 2006). Benthic diatoms play a pivotal role in the functioning of estuarine ecosystems; they provide up to 50% of the primary production of estuaries (Underwood & Kromkamp 1999), contribute to sediment stabilization through the production of extracellular polymer secretions (Decho 2000) and influence nutrient fluxes between sediment and water (Sundback et al. 2000).

A field study by Forster et al. (2006) on intertidal mudflats in the Westerschelde estuary (The Netherlands) showed a positive relationship between net primary production and diversity of natural
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Diatom communities. We further explored this relationship by composing experimental assemblages of naturally co-occurring diatom species with nonrandomly decreasing diversity and analysed the effect of species richness and composition on biomass production. Biodiversity effects were assessed by measuring overyielding (occurring when a mixture of species performs better than its monocultures). The mechanisms behind the biodiversity effects were explored by partitioning the biodiversity effects using the additive partitioning equation of Loreau & Hector (2001) and by conducting an additional culture experiment to elucidate potential facilitative interactions between different species.

Material and methods

Biodiversity-ecosystem functioning experiment

The effect of diatom species richness on community yield was assessed using eight benthic diatom species (Table 1). These species were isolated from the 'Paulina' intertidal mudflat in the Westerschelde Estuary, The Netherlands (51°21' N, 3°43' E) where they are dominant components of the microphytobenthos (Sabbe & Vyverman 1991; Forster et al. 2006). Voucher slides of the cultures used for the experiments were kept in the Laboratory of Protistology and Aquatic Ecology, Ghent University, Belgium.

In total, we assembled 19 species combinations spanning four species richness levels (one, two, four and eight species, combinations shown in Table 1) in a replicated combinatorial design (Giller et al. 2004) which replicated both species richness and species composition, with five replicates per treatment. We replicated three species levels regarding species composition (one, two and four species); the eighth species community was only the pool of species used here. Therefore, we cannot separate the effect of species number and identity on the production for the eighth species level. Although this is a serious drawback, we included this mixture because it can shed some light on the interspecific interactions when all species are combined.
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Statistical analyses

We used the additive partitioning method (Loreau & Hector 2001) to calculate the net biodiversity, selection and complementarity effects. The net biodiversity effect of a mixture was calculated as the difference between the observed yield and the expected yield (weighted average of the monocultures), under the null hypothesis that there was no selection or complementarity effect. The net biodiversity effect could be partitioned into two additive components: the selection effect and the complementarity effect. The complementarity effect for a specific number of species N was N \( \Delta R Y M \), where \( \Delta R Y \) is the average change in relative yield for all species in the mixture and \( M \) is the average monoculture yield. The selection effect N \( \cdot \text{cov} (\Delta R Y, M) \) was calculated as the covariance between the monoculture yield of species (M) and their change in relative yield in the mixture (\( \Delta R Y \)) multiplied by the number of species in the mixture (N). Positive selection occurred when species with high monoculture yield dominated the mixtures, while negative selection arose when species with low monoculture yield dominated or when species with high monoculture yield performed worse in mixtures. Negative selection effects were documented in seagrass communities where plant mortality in mixtures was reduced in genotypes that were weak in monoculture (Reusch et al. 2005). Another example is found in serpentine grasslands where perennial bunchgrass with high monoculture yields produced substantially less biomass in mixtures with other functional groups (Hooper & Vitousek 1997). As our data were not normally distributed, we used the Spearman rank method for calculating the correlation (rho) of species richness with biovolume yield, net biodiversity effects, complementarity and selection effects (R Development Core Team 2005).

We evaluated the effects of species composition (identity effect) and species richness (diversity effect) on biomass yield using a generalized linear mixed model (GLMM) with species richness as a fixed factor and species composition as a random factor nested within species richness. We treated species composition as a random factor because replicate species compositions were only a small sample of all possible species combinations. Species richness was treated as a fixed factor because its levels are deliberately selected and form a larger fraction of the possible range. As the standard deviations of the different combinations were
correlated with the average yield, we log-transformed the yield values. The variances between the different combinations differed between species richness levels. Therefore, a GLMM with the variances estimated for each species richness level was used. The link function was "proc mixed" and the distribution family was normal. The variance structure was "variance components".

We verified which mixtures outperformed their most productive component monoculture by calculating the transgressive overyielding measure $D_{\text{max}}$ (Loreau 1998). We assessed the performance of individual species in each mixture by considering the proportional deviation ($D_i$) of species $i$'s yield from its expected value based on its monoculture yield (Loreau 1998).

**Results**

After 25 days, the initial biovolume of $4.78 \cdot 10^7 \, \mu m^3$ algal cells added to the experimental microcosms significantly increased in all treatments. Analysis of diatom biovolume (Fig. 1a) of the different species assemblages revealed an increase in yield with species richness (Spearman rank correlation rho of 0.54, $P = 0.016$). The transgressive overyielding measure $D_{\text{max}}$, which indicates when a mixture outperforms its most productive component monoculture, was positive in 6 out of 11 treatments.

The GLMM (Table 2) confirmed that species richness influenced biomass yield ($F = 4.59, P = 0.018$) and showed that the identity of the monocultures significantly influenced their production ($Z = 1.86, P = 0.0312$) but was unable to show significant differences in yield between different combinations within higher species richness levels. A Tukey-Kramer post hoc test showed significant differences in yield between communities of four species and monocultures (adjusted $P = 0.0422$).

The net biodiversity effect (Loreau & Hector 2001) increased with species richness (Fig. 1b, Spearman rank correlation of 0.82, $P = 0.002$). The biovolume data for the individual species in the assemblages allowed us to separate the net biodiversity effect into a selection and a complementarity effect (sensu Loreau & Hector 2001). The selection effect showed a decreasing trend and became negative with increasing species
richness (Fig. 1d), which indicated that assemblages with higher species richness were dominated by species with low monoculture yields. The negative selection effects in treatments one, three, four, five and nine were mainly caused by the dominance of *C. closterium* and/or *Nitzschia* sp., wherein the yields in the above mixtures exceeded their monoculture yields. The complementarity effect was positive for all except one treatment (seven) and increased with species richness (Fig. 1c).

**Figure 1.** Effects of diatom diversity on (a) biomass yield, (b) net biodiversity effect (the difference between the observed yield of the mixture and the expected yield, Loreau & Hector 2001), (c) complementarity effects and (d) selection effects (sensu Loreau & Hector 2001). Arrows indicate the treatments where transgressive overyielding ($D_{max}$) occurred. The numbers denote the different treatments as defined in Table 1. Open circles represent treatments without *Cylindrotheca closterium*, filled circles represent treatments which contain *C. closterium*. 

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Table 2. Results of a generalized linear mixed model (GLMM) with species richness as a fixed factor and species composition as a random factor nested within species richness. Variances were estimated for each species richness level. Abbreviations: comb: species combinations, SRL: species richness level, SE: standard error, d.f.: degrees of freedom, n.a.: not applicable. Diff.: difference.

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<td>1.2378 (0.6053)</td>
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<td>Species Combinations in SRL 2</td>
<td>0.06049 (0.04137)</td>
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<td>Species Combinations in SRL 8</td>
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<td>Species richness</td>
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Tukey-Kramer post hoc test

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<td>Diff SRL 1 - 4</td>
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The relation between the performance of individual species (D_i), and the number of species in the assemblage is shown in Fig. 2. A positive correlation was observed for C. closterium and N. gregaria (Fig. 2a,b), while N. arenaria, N. flanatica, N. cincta and H. crucigera showed a neutral to negative relation (Fig. 2e-h). The D_i of Nitzschia sp. and of N. phyllepta (Fig. 2c,d) showed a nonlinear relation with species richness, indicating that the species composition rather than its richness influenced the performance of Nitzschia sp. The proportional deviation of the other species was not significantly correlated with species richness.

To test whether facilitation between different diatom species was the mechanism that promoted the growth of C. closterium in multispecies assemblages (see D_i of this species in Fig. 2a), we conducted an additional experiment assessing the growth of a C. closterium monoculture in an environment where there was no influence of other diatom species and in an environment where we added spent medium of different Navicula
species. We observed a disproportionate threefold increase in cell numbers when we added spent medium and, surprisingly, a drastic six fold decrease in chlorophyll a content per cell (Fig. 3).

**Figure 2.** Proportional deviation Di (Loreau 1998) of species yield from its expected value (a): Cylindrotheca closterium, (b): Navicula gregaria, (c): Nitzschia sp., (d): Navicula phylepta, (e): Navicula arenaria, (f): Navicula flanatica, (g): Haslea crucigera, (h): Navicula cincta.

**Figure 3.** Biovolume yield (a) and chlorophyll a yield (b) of a monoculture of Cylindrotheca closterium when grown in inorganic 'f/2' medium and in 'f/2' medium enriched with spent medium of a mixture of Navicula species. (c): boxplot of the chlorophyll a to biovolume ratio in both treatments.
Discussion

Our results demonstrated strong effects of non-random diversity changes on biomass production in experimentally assembled benthic diatom communities. Positive diversity effects could largely be attributed to strong positive complementarity effects (incorporating both niche complementarity and facilitation). At the highest diversity (eight species) the shape of the species richness-net biodiversity effect curve levelled off. This was due to negative selection effects which partly counteracted the positive complementarity effects and was mainly caused by the dominance of species with low monoculture yields (particularly *C. closterium* and *Nitzschia* sp.). Negative selection effects seem to be relatively common in BEF experiments (Fargione *et al.* 2007; Jiang 2007) and can significantly decouple effects of complementarity from net effects on production.

The overall effects of biodiversity on biomass production were highly positive, especially when mixture yields were compared with monoculture yields. Six out of 11 combinations outperformed their most productive component monoculture. This phenomenon, called transgressive overyielding, is a strong indication for niche complementarity and/or facilitation, since sampling effects alone cannot cause this type of overyielding (Drake 2003). The frequent occurrence of transgressive overyielding (in 54% of our mixtures) was remarkable as most BEF studies demonstrated very low frequencies or absence of transgressive overyielding (Hector *et al.* 2002; Drake 2003; Hooper & Dukes 2004; Cardinale *et al.* 2006). The few studies that did observe transgressive overyielding were long-term experiments (covering 10 generations, Fargione *et al.* 2007), showing that positive diversity effects might increase over time. Hector *et al.* (2002) suggest that the conditions where resource partitioning and positive feedback mechanisms become apparent were not yet fully developed in many short-term experiments (in terms of number of generations) using higher plants. Our experiment was conducted with rapidly dividing microbial species (covering five to six generations), which may explain the observed high frequency of transgressive overyielding. In addition, the fact that we simulated non-random species loss, which often results in more rapid and disproportionate loss of function due to faster disappearance of functional
groups (Solan et al. 2004; Zavaleta & Hulvey 2004; Bracken et al. 2008) might also have led to more pronounced biodiversity effects.

The high frequency of transgressive overyielding in our experiments (in 54% of the mixtures) can be further explained by the effects of species richness on production and the underlying mechanisms mediated by specific environmental conditions (Cardinale et al. 2000). Facilitation, one of the mechanisms potentially leading to overyielding, is considered to be more important under harsh environmental conditions (Bertness & Leonard 1997; Mulder et al. 2001). Harsh limiting conditions, such as desiccation, highly variable salinity and temperature and regular physical disturbance are constantly encountered by diatoms of intertidal mudflats and could be alleviated through the intimate association of cells in biofilms which offers shelter against bottom shear stress from wave action. This physical protection is strengthened by positive feedback interactions between diatom growth and decreased sediment erosion (Van de Koppel et al. 2001). Here we demonstrated that diversity per se (and the increasing probability of positive interactions) could thus maximize net production of biofilms which might reinforce the reported positive feedback mechanisms between biofilm production and decreased sediment erosion. Future research should be conducted on the effects of species diversity on biofilm stability.

The observed positive diversity effects were largely attributable to strong positive complementarity effects which could be caused by niche complementarity and/or facilitation. At the moment we do not have insights into mechanisms of niche complementarity but it is conceivable that there is complementary use of light and/or nutrients. We did find facilitative interactions when we calculated the difference between the yield of a particular species in the presence of other species and as a monoculture. We found that the yield of *C. closterium* strain VD18 and *Nitzschia* sp. was almost always strongly promoted in the presence of other diatom species, indicating facilitation (Hodgson et al. 2002). While this suggests that facilitation is an important component of the observed complementarity, we cannot infer exactly to what extent facilitation contributed to the positive diversity effect in our experiment. Although *Nitzschia* sp. generally benefited from the presence of other diatom species, its performance decreased in the eight species mixtures (Fig. 2c). In this case, interspecific competition was probably stronger than the facilitative interactions. Some caution is warranted, however, when drawing conclusions from the eight species level, because the species
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composition at this diversity level is not replicated. Because of this, species identity effects cannot be separated from species diversity effects for this species richness level. The results of additional experiments with *C. closterium* strain VD18 confirmed the occurrence of facilitation. The growth (in terms of cell numbers) of *C. closterium* monocultures was strongly promoted when spent medium of a different *Navicula* species was added, while the cellular chlorophyll a content showed a sixfold decrease. Since the *Navicula* species modified the environment in a way favourable to *C. closterium* cells, we can conclude that facilitation occurred (Vandermeer 1989; Fridley 2001). Previous experiments with *C. closterium* strain VD18 showed that the culture medium (f/2) was sufficiently nutrient rich that even half strength did not depress growth rate over similar periods of time and under identical culture conditions, implying that inorganic nutrients should never be limiting to *C. closterium* strain VD18 in the additional experiment (B. Vanelslander, unpublished data). On the other hand, we previously found that the growth of this strain improved when galactose was added to the culture medium, showing the mixotrophic capacities of this strain. Mixotrophic growth of microalgae is known to cause a decrease in specific chlorophyll a content and reduction or even loss of photosynthetic capacity (Tittel et al. 2005). Considering the mixotrophic capacities of this strain and the absence of inorganic nutrient shortage, we concluded that the improved growth and concomitant decrease in chlorophyll a content was caused by a shift from photoautotrophic to mixotrophic growth on exudates of other diatom species, potentially modified by bacteria. In contrast, two other *C. closterium* strains (VD05 and VD06) incapable of using galactose to improve their growth did not show significantly differing growth rates and had an unaltered chlorophyll a content when the same spent medium of different *Navicula* species was added (B. Vanelslander, unpublished data). These observations strengthened our conclusion that the growth of *C. closterium* strain VD18 was promoted by the availability of organic exudates in the spent medium. Benthic environments are often characterized by frequent light limitation due to burial and shading by thick algal mats and by relatively high concentrations of dissolved organic compounds (Cook et al. 2004). These conditions provide a competitive advantage to phototrophic cells which have the ability to take up dissolved organic substrates as a carbon or nitrogen source (Linares & Sundback 2006). Laboratory studies have shown that the growth of a number of benthic microalgae (including *C. closterium*) was enhanced by
addition of dissolved organic substrates, ranging from monomeric sugars to complex organic molecules (Hellebust & Lewin 1977; Tuchman et al. 2006). The importance of mixotrophic growth in the nutritional ecology of benthic microalgae is almost unexplored. Our results indicate that mixotrophic capacities of microalgae can cause facilitative interactions, and that these interactions can contribute to the observed positive effects of biodiversity on biomass production in intertidal microphytobenthic communities.

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References


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Chapter 5


Diversity-Productivity in benthic diatom biofilms


Chapter 5


Chapter 6

Interference Competition between Marine Benthic Diatoms

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Abstract

Spatial variation in species composition of benthic microalgae communities is poorly understood. Variation in community composition over large spatial scales is typically related to gradients in environmental parameters. Yet, at smaller spatial scales biofilms of microalgae often show patchy distribution patterns even when there is little or no obvious abiotic heterogeneity. While it can be envisaged that colonisation dynamics and local biotic interactions are able to generate such micropatchiness, little is know about the nature and importance of these mechanisms. In this study we use co-culture experiments to demonstrate that estuarine benthic diatoms differ strongly in their capacity for interference competition. We show that cell cultures of the marine benthic diatom *N. cf. pellucida* exude metabolites with strong allelopathic effects. All nine competitor species tested were inhibited in their growth and some in their photosynthetic efficiency as well. In addition, we show reciprocal, density dependent allelopathic interactions between *N. cf. pellucida* and two other marine benthic diatom species, *Entomoneis paludosa* and *Stauroonella* sp. Our results suggest that allelopathic interactions might be common in benthic microalgal communities and may explain small-scale spatial distribution patterns observed in nature.
Introduction

Estuarine intertidal mudflats are characterized by steep gradients in light, nutrients and oxygen and hence there is strong competition for limiting resources among microphytobenthic algae (Admiraal, 1984). In temperate regions, the intertidal microphytobenthos is dominated by diverse diatom communities. These diatom assemblages display pronounced spatial and temporal variation in species composition. Environmental parameters such as sediment type, temperature, light, salinity, ammonium concentration, etc explain much of the large-scale rates of community turn-over in microphytobenthos (Thornton et al., 2002, Du et al., 2009, Underwood, 1994). However, at centimetre scales, the biomass and species composition of intertidal benthic diatoms sometimes display spatial patterns (Shaffer & Onuf, 1985, de Brouwer et al., 1999), with different species showing a complementary spatial arrangement with non-overlapping areas of maximal density (Saburova et al., 1995). The negative correlation between the abundances of different species could be due to microscale habitat heterogeneity and/or biological interactions, including species specific grazing (Smith et al., 1996, Hagerthay et al., 2002, Hamels et al., 2004) and interspecific competition. Evidence is accumulating that microalgae are able to engage in interference competition as a means to acquire resources (Gross, 2003, Legrand et al., 2003).

In phytoplankton, density dependent allelopathy, the release of chemicals that inhibit the growth or survival of competing species, has been shown to be an important determinant of species composition and succession in phytoplankton (Keating, 1977, Vardi et al., 2002, Kabanek et al., 2005), and affects the initiation and/or continuation of plankton blooms (Smayda, 1997, Legrand et al., 2003, Prince et al., 2008a). Such chemically mediated interference competition has been demonstrated for phytoplankton taxa from groups as divergent as dinoflagellates, chlorophytes, diatoms and cyanobacteria (Gross, 2003, Legrand et al., 2003, Poulson et al., 2009). In many cases, the chemical nature of allelopathic compounds is unknown, but several modes of action of allelochemicals on susceptible target cells have been demonstrated. These include inhibition of Photosystem II efficiency (Gross, 2003, Prince et al., 2008a), membrane damage (Legrand et al., 2003), inhibition of enzymes (Sukenik et al., 2002), reduced motility (Uchida et al., 1999) and
oxidative damage (Legrand et al., 2003, Prince et al., 2008a, Poulson et al., 2009).

In benthic phototrophic biofilms, the intimate association and high density of cells enables strong chemical communication and cell-cell interactions between microbial cells (Decho, 2000). Allelopathic interactions have been demonstrated for several benthic cyanobacteria (Mason et al., 1982a, Gross et al., 1991, Juttner, 1999, Gross, 2003), but there is less evidence for eukaryotic benthic microalgae. De Jong and Admiraal (1984) observed that cells of the estuarine benthic diatom *Cylindrotheca closterium* died in mixed cultures with *Entomoneis* cf. *paludosa*. Another marine diatom, *Haslea ostrearia*, produces a blue-green polyphenolic pigment, marenine, that inhibits the growth of naturally co-occurring microalgae (Pouvreau et al., 2007). Leflaive and Ten-Hage (2009) showed that menthanolic cell extracts (so not only exuded metabolites) of *Uronema confervicolum* and *Desmodesmus quadrispina* inhibited the growth of several microalgae.

In this study we document that the diatom *Nitzschia* cf. *pellucida* inhibits the growth of several key epipsammic and epipelic diatom species of intertidal microphytobenthos of the Scheldt Estuary. We further show the occurrence of dependent mutual allelopathic interactions between *N. pellucida* and two other marine benthic diatom species, *Entomoneis paludosa* and *Stauronella* sp.

**Material and Methods**

**Strain isolation and culture conditions**

All diatom strains were isolated from the “Rammekenshoek” intertidal mudflat in the Westerschelde Estuary, The Netherlands (51°26’50” N, 3°38’38” E) on March 03, 2009 except CCY 9601and IID03 which originate from Ems Dollard (Holland) and Doel (Belgium). Voucher slides of the cultures used for the experiments are kept in the Laboratory of Protistology and Aquatic Ecology, Ghent University, Belgium. The identification of the diatom strains was based on Krammer and Lange-Bertalot (1986-1991).
Clonal cultures were established as described in Chepurnov et al. (2002). Culture medium was composed of filtered and autoclaved North Sea seawater enriched with f/2 nutrients (Guillard, 1975). Clonal cultures were maintained in a climate room at 18 ± 1 °C and illuminated by cool-white fluorescent lamps at a rate of 50 μmol photons m⁻² s⁻¹ with a light/dark cycle of 12/12 hours. The experiments described below were performed under the same conditions.

**Co-culture experiments**

Preliminary experiments with co-cultures of diatom species revealed that the diatom *Nitzschia cf. pellucida* exerted strong adverse effects on other diatom species. We therefore focused on the growth interactions between *N. cf. pellucida* and 9 benthic diatom species belonging to various genera (*Gyrosigma* sp, *Navicula arenaria* Donkin, *Nitzschia* sp., *Stauronella* sp., *Cylindrotheca fusiformis* Reimann & Lewin strain CCY 9601, *Cylindrotheca fusiformis* (Ehrenberg) Reimann & Lewin strain IID03, *Opephora* sp, *Amphora* sp, and *Leyanella arenaria* Hasle, von Stosch & Syvertsen). Competition experiments were performed in polystyrene 24-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany) containing 2.0 mL f/2 culture medium. Each species was inoculated at a cell density of ~ 5,000 cells mL⁻¹ resulting in an initial density of ~ 50 cells mm⁻². Uni-algal treatments were included as control and inoculated at 10,000 cells mL⁻¹. Cells for inoculations were obtained from monoclonal, exponentially growing cultures. Each treatment was replicated four times. The culture medium was renewed daily (1.0 mL of culture medium was replaced with 1.0 mL of double strength f/2 medium) to minimise nutrient limitation and hence resource competition for nutrients. The cell density of each species in all replicates was monitored daily using an inverted microscope (Axiovert 135 Zeiss microscope, Jena, Germany) by counting a minimum of 300 cells per replicate. Growth rate during the exponential phase (4-5 days) was calculated as the slope of the linear regression of log₂ transformed cell densities versus time for individual cultures.

To assess the importance of direct cell contact for allelopathic interactions, we co-cultured *Navicula arenaria*, *Cylindrotheca fusiformis* IID03, *Gyrosigma* sp. (three of the most susceptible species, see below) and *Stauronella* sp. together with *N. cf. pellucida* but separated from each other by a 1 μm pore size membrane filter using cell culture inserts (Thin
We inoculated 20,000 cells of each species (~50 cells/mm$^2$ in the outer chamber and ~177 cells/mm$^2$ in the inner chamber). *N. cf. pellucida* cells were cultured in the outer chamber, the second species in the inner chamber. As a control, we replaced the *N. cf. pellucida* culture with fresh F/2 medium. Each treatment was replicated four times. The culture medium was renewed daily (2.0 mL of double strength f/2 medium). We monitored growth and photosynthetic efficiency by pulse amplitude modulated (PAM) fluorescence (MAXI Imaging PAM fluorometer, Walz, Germany). We used the maximum quantum yield of Photosystem II (PSII) as a proxy for photosynthetic efficiency (Kromkamp et al., 1998). This was measured as $F_v : F_m$, with $F_v = F_m - F_o$ (Parkhill et al., 2001). $F_m$ is the maximum fluorescence emission level in the dark measured with a saturating pulse of light (emission peak at 450 nm, 2700 µmol photons·m$^{-2}$·s$^{-1}$, 800 ms). The initial fluorescence, $F_o$ was used as a proxy for biomass (Honeywill et al., 2002).

From preliminary experiments and from the experiment shown in Fig. 1, we suspected reciprocal allelopathic interactions for the combinations of *N. cf. pellucida* with *E. paludosa* and *Stauronella* sp. Therefore we conducted bi-algal culture experiments with different cell densities and ratios of these competing species. We inoculated these species with total initial cell densities of 10,000 and 30,000 cells mL$^{-1}$ and with the initial ratios between the two species of 1:4; 1:1, 4:1.

**Results**

Between 1 and 4 days, the growth of the 9 tested benthic diatom species was first suppressed and then arrested in the presence of *Nitzschia* cf. *pellucida* and eventually all cells of these species suffered cell lysis and death (Fig. 1). The cell density at which *Nitzschia* cf. *pellucida* was inhibiting and lethal was species specific. *Gyrosigma* sp., *Navicula arenaria* and *Cylindrotheca fusiformis* strain IID03 were the most sensitive strains as they were inhibited and died after 1 day of exposure to relatively low densities of *N. cf. pellucida* (between ~7500 to 9500 cells mL$^{-1}$). *Entomoneis paludosa*, *Cylindrotheca fusiformis* strain CCY 9601, *Stauronella* sp., and *Amphora* sp. were inhibited after 2 to 4 days.
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exposure (at \(N. \text{ cf. pellucida}\) densities of between \(-30,000\) and \(150,000\) cells \(\text{mL}^{-1}\)) and died soon after, while \(Leyanella \text{ arenaria}\) and \(Opephora\) spp. were more resistant and died gradually with an almost complete extinction after approximately 10 days.

The growth rate of \(N. \text{ cf. pellucida}\) did not differ significantly in the presence or absence of other species (paired two-tailed t-test, \(p > 0.1\)), except when grown together with \(Stauronella\) sp. \((p = 0.008)\), which significantly decreased the growth of \(N. \text{ cf. pellucida}\) but still died after 4 days.

Figure 1. Growth interactions between \(Nitzschia \text{ cf. pellucida}\) and 9 other benthic diatom species belonging to 8 different genera. Species were cultured alone (grey triangles, dashed lines) and together (grey open circle, solid lines) with \(N. \text{ cf. pellucida}\) (filled black square, solid lines). \(N. \text{ cf. pellucida}\) monocultures were included (open black squares, dashed lines). Target species were \(Gyrosigma\) sp. (A), \(Navicula\) arenaria (B), \(Entomoneis paludosa\) (C), \(Stauronella\) sp. (D), \(Cylindrotheca \text{ fusiformis CCY 9601}\) (E), \(C. \text{ fusiformis IID03}\) (F), \(Opephora\) sp (G), \(Amphora\) sp (H) and \(Leyanella \text{ arenaria}\) (I). Error bars are standard deviations.
Thin Cert experiments with selected species combinations showed broadly similar, but on average less pronounced effects of *N. cf. pellucida*. The growth of *N. arenaria* was significantly affected after 4 days; after 6 days biomass estimated by fluorescence was 39.2% lower than in monocultures, at a *N. cf. pellucida* cell density of ~180,000 cells/mL. The photosynthetic efficiency (Fv : Fm) showed only a weak decrease (-17 %). Cells of *C. fusiformis* strain IID03 were affected after 4 days and after 5 days biomass was 56 % lower than in monocultures (at *N. cf. pellucida* cell densities of ~160,000 cells mL⁻¹). The photosynthetic efficiency decreased with 52.0 % after 5 days compared to the monocultures. The growth of *Stauronella* sp. was already affected after 2 days and after 5 days biomass was 36.9% lower than in monocultures (at a *N. cf. pellucida* cell density of ~160,000 cells mL⁻¹). Its photosynthetic efficiency however was unaffected. The growth and photosynthetic efficiency of *Gyrosigma* sp. were not affected at all after 6 days indirect exposure to *N. cf. pellucida* cells.

Figure 2. Co-cultivation of *N. cf. pellucida* with 4 other diatom species but separated by a 1 µm membrane filter. Biomass (A-D) (measured as initial fluorescence F0) and photosynthetic efficiency (E-H) (Fv : Fm measured by PAM fluorescence) of *Navicula arenaria* (A,E), *Cylindrotheca fusiformis* IID03 (B, F), *Stauronella* sp. (C, G) and *Gyrosigma* sp. (D, H) in monocultures (triangles and dashed lines) and together with *N. cf. pellucida* but separated by a 1µm pore size membrane (squares and solid lines). Error bars are standard deviations.
Based on the *N. cf. pellucida* vs *Stauronella* sp. co-culture experiment shown in Fig. 1 and on preliminary experiments with *E. paludosa* and *N. cf. pellucida*, we suspected the occurrence of reciprocal, density-dependent allelopathic interactions. When we varied the (relative) starting densities of *N. cf. pellucida* and *Stauronella* sp., we indeed observed reciprocal inhibition between both species and this depended strongly on their respective cell concentrations (Fig. 3 and 4). When *Stauronella* sp. cell numbers reached ~100,000 cells mL\(^{-1}\), *N. cf. pellucida* growth was suppressed. Yet, when *N. cf. pellucida* reached ~80,000 cells mL\(^{-1}\), *Stauronella* sp. cell numbers declined. Similar reciprocal density-dependent effects were seen between *N. cf. pellucida* and *E. paludosa*. *E. paludosa* cell numbers declined when *N. cf. pellucida* reached cell densities of ~50,000 - 70,000 cells mL\(^{-1}\). On the other hand, when *E. paludosa* reached a cell density of ~100,000 cells mL\(^{-1}\), growth of *N. cf. pellucida* was suppressed, again in the presence of an adequate nutrient supply.

**Figure 3.** Growth interactions between *Nitzschia* cf. *pellucida* and *Entomoneis paludosa* at different initial cell densities. Species were cultured alone (dashed lines) and together (solid lines). Cell densities of *N. cf. pellucida* represented by black lines, *E. paludosa* cell densities represented by grey lines. (A-C): total initial cell density was 10,000 cells mL\(^{-1}\) with *N. cf. pellucida* : *E. paludosa* initial cell density ratio's of 1:4 (A), 1:1 (B), 4:1 (C). (D-E) total initial cell density at 30,000 cells mL\(^{-1}\) with *N. cf. pellucida* : *E. paludosa* initial cell density ratio's of 1:4(D), 1:1 (E), 4:1 (F). Error bars are standard deviations.
Figure 4. Growth interactions between *Nitzschia* cf. *pellucida* and *Stauronella* sp. at different initial cell densities. Species were cultured alone (dashed lines) and together (solid lines). Cell densities of *N*. cf. *pellucida* represented by black lines, *Stauronella* sp. cell densities represented by grey lines. (A-C): total initial cell density was 10,000 cells mL⁻¹ with *N*. cf. *pellucida* : *Stauronella* sp. initial cell density ratio’s of 1:4 (A); 1:1 (B), 4:1 (C). Error bars are standard deviations.

Discussion

We show that the diatom *Nitzschia* cf. *pellucida* inhibits the growth of and ultimately kills all nine tested benthic diatom species. These competitors naturally co-occur with *N*. cf. *pellucida* and most of them are common in European intertidal mudflats. Several of the diatom species tested (*Gyrosigma* sp., *N*. arenaria, *C*. fusiformis, *E*. paludosa) were inhibited at relatively low cell densities (~ 8,000 – 10,000 cells mL⁻¹ equivalent with ~ 8,000 – 10,000 cells cm⁻²) of *N*. cf. *pellucida*. It seems unlikely that nutrient depletion would cause the observed growth inhibition as most inhibition and cell death occurs already in the first 3 days (fig. 1). In contrast, cells of *N*. cf. *pellucida* in the co-cultures display exponential growth during the first 5 days, implying nutrient replete conditions during the first days of the co-culture experiments.

The observed patterns of allelopathic interactions suggest that in natural epipelic diatom communities these negative interactions may operate and influence community composition, given that diatoms can reach total cell densities of 1.10⁶ cells cm⁻² in estuarine mudflats (Admiraal et al., 1982). Whereas *N*. *pellucida* is a cosmopolitan species inhabiting marine coasts and is commonly found worldwide (Krammer & Lange-Bertalot, 1986-1991), it is currently unknown whether all strains
(or lineages) of this morphospecies produce allelochemicals and thus how widespread this mechanism occurs.

However, effects of co-cultivation in different compartments separated by a 1µm pore size membrane are less pronounced than in co-cultures where cells of the two species were completely mixed. This may indicate that close cell-cell contact or contact with the extracellular polymeric saccharides (EPS) matrix enhances the allelopathic interactions and/or that the allelopathic compound is volatile and/or little soluble in water. Close proximity of competing algae and cell-cell contact may have a large contribution to the effectiveness of allelochemicals since toxin concentration in close proximity of a cell can be many times higher than further away from the cell (Jonsson et al., 2009). Transfer of more hydrophobic allelochemicals by direct cell-cell contact is highly conceivable in microalgal biofilms where cells are densely packed in a polymeric matrix (Decho, 2000). Several surface-associated allelopathic interactions have been demonstrated for benthic cyanobacteria such as Fischerella sp. (Gross, 1999) and Scytonema hofmanni (Mason et al., 1982b), but only very few for eukaryotic benthic microalgae. Interestingly, three of the five most resistant diatom species were non-motile (Leyanella arenaria, Amphora sp. and Opephora sp.) epipsammic diatoms which live attached to sand grains. In contrast, the more susceptible diatom species were all epipelagic diatoms which exhibit a motile lifestyle and thus have higher probabilities for physical encounters with cells of N. cf. pellucida but are also able to escape them.

N. cf. pellucida exudates can diminish the photosynthetic efficiency of competing algae, as was shown in our Thin Cell co-cultivation experiments. Several studies already demonstrated decreased PSII efficiency due to allelopathy (Legrand et al., 2003, Prince et al., 2008a), but whether PSII is really the target of allelopathic compounds or if decreased efficiency is merely a symptom of deteriorating cell functioning is usually unknown. Sukenik et al. (2002) for instance showed that Microcystis exudates inhibited photosynthesis of Peridinium gatunense. This was not the result of direct effects on the photosystems, but due to a strong suppression of internal carbonic anhydrase which caused a reduced availability of CO₂ and thus a reduced photosynthesis.

Allelochemicals can be very effective, but the target cells are not necessarily suffering passively and may try to escape through the formation of temporary cysts (Uchida et al., 1999, Fistarol et al., 2004). Competitors might also counteract allelopathy by degrading
allelochemicals (Prince et al., 2008b) or can be constitutively resistant towards specific allelochemicals (Kubanek et al., 2005). Still others can produce their own allelopathic compounds as a response on allelochemicals (Vardi et al., 2002). Furthermore, relative abundance can determine the outcome of allelopathic interactions as suggested by our experiments using different initial densities of \textit{N. cf. pellucida} and \textit{E. paludosa} or \textit{Stauronella} sp. in co-culture experiments. The species which first reached a certain cell density threshold could suppress growth of the other species resulting in considerable mortality, even with ample nutrient supply. Basically, this phenomenon can be described as a priority effect which is a lasting impact of the order of arrival of species on the community development (Van Gremberghe et al., 2009). Priority effects can be caused by two different mechanisms, firstly, a numerical effect in which the first species can reach carrying capacity before other species arrive. Secondly, species can alter their environment in a favourable or detrimental way for later colonizing species (Van Gremberghe et al., 2009), which is what we observed in our culture experiments.

Our observation that \textit{E. paludosa} is able to exude growth inhibiting metabolites confirms the preliminary observations by De Jong and Admiraal (1984) on a \textit{E. cf. paludosa} strain. Reciprocal, density dependent allelopathic interactions between the planktonic dinoflagellate \textit{Peridinium gatunense} and the cyanobacterium \textit{Microcystis} sp. in the Sea of Galilee were proposed to promote the observed patchy distribution patterns of these species in the lake (Vardi et al., 2002). Similarly, in microphytobenthos such chemical cross-talk may also help to understand observations of patchy spatial occurrence of microphytobenthos species at small (cm) spatial scales, and are in agreement with the findings of Saburova et al (1995) who showed that different species showed complementary spatial arrangement with non-overlapping areas of maximal densities, but only when threshold densities of interacting species were reached.

Overall, we demonstrate that the marine benthic diatom \textit{N. cf. pellucida} produces strong allelochemicals that kill all tested diatom species. We further show reciprocal, density dependent allelopathic interactions between \textit{N. pellucida} and two other marine benthic diatom species, \textit{Entomoneis paludosa} and \textit{Stauronella} sp. Our results suggest that allelopathic interactions might be common in benthic microalgal
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communities and may influence their small-scale spatial distribution patterns.

Cited References


Interference competition in benthic diatoms


Chapter 7

The "Molecular Toothbrush" of a Microalga: Daily Bursts of Biogenic Cyanogen Bromide (BrCN) Control Biofilm Formation around a Marine Benthic Diatom

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Abstract

The spatial organization of biofilms is strongly regulated by chemical cues released by settling organisms. Yet, the exact nature of these interactions and the repertoire of chemical cues and signals that micro-organisms produce and exude in response to the presence of competitors remain largely unexplored. Biofilms dominated by microalgae often show remarkable, yet unexplained fine-scale patchy variation in species composition. Since this occurs even in absence of abiotic heterogeneity, antagonistic interactions might play a key role. Here we show that the marine benthic diatom *Nitzschia* cf. *pellucida* exudes a diverse mixture of volatile iodinated and brominated metabolites including the new natural product cyanogen bromide (BrCN) which exhibits pronounced allelopathic activity. Toxin production is light-dependent with a short toxin burst after sunrise. This labile compound acts as a short-term signal, leading to daily “cleaning” events around the algae. We show that allelopathic effects are H$_2$O$_2$ dependent and link BrCN production to haloperoxidase activity. This novel strategy is a highly effective means of biofilm control and provides an explanation for the poorly understood role of volatile halocarbons from marine algae which contribute significantly to the atmospheric halocarbon budget.
Introduction

Biofilm formation in marine habitats is a rapid and ubiquitous process and most submerged surfaces, natural or man-made, are covered with complex microbial communities. Intense efforts are made to control biofilm formation on industrial surfaces such as ship hulls because this biofouling can result in severe economic loss (Yebra et al., 2004). Among the early settlers, microalgae play a key role in the biofilm development and diatoms, especially, are able to settle on even the most fouling resistant surfaces (Molino & Wetherbee, 2008). In this context it is interesting to observe that certain microalgae can obviously control their microenvironment since the patchy variation in species composition observed around these algae (Shaffer & Onuf, 1985, Saburova et al., 1995, de Brouwer et al., 2000) cannot be explained by abiotic heterogeneity or by grazers because the production of microalgae in dense biofilms far exceeds the total consumption, making the effect of grazing limited (Saburova et al., 1995). This spatial organization of species is characterized by complementary distribution patterns and negative correlation of species densities (Saburova et al., 1995). Allelopathic interactions have been suggested as a possible explanation for such observed patchiness (Saburova et al., 1995). Because biofilms are composed of densely packed cells embedded within a matrix of exuded polymeric compounds, metabolites produced by any cell can efficiently target its neighbours rather than diffusing into the surrounding water column (Decho, 2000).

Studies that focused on interspecific interactions between biofilm-forming diatoms revealed that synergistic (Vanelslander et al., 2009) and antagonistic (De Jong & Admiraal, 1984, Chapter 6) interactions are common and can have a strong influence on biofilm performance (Vanelslander et al., 2009). The underlying chemistry of these interactions is unknown, but several modes of action of allelochemicals on susceptible target cells have been demonstrated, including the inhibition of photosynthesis (Gross, 2003, Prince et al., 2008), membrane damage (Legrand et al., 2003), inhibition of enzymes (Sukenik et al., 2002), reduced motility (Uchida et al., 1999) and oxidative damage (Legrand et al., 2003, Prince et al., 2008, Poulson et al., 2009). In this study we selected the common biofilm forming diatom Nitzschia cf. pellucida due to
the high allelopathic activity observed in chapter 6 of this PhD. Several *Nitzschia* species are known for their production of volatile halocarbons (Sturges *et al*., 1993, Moore *et al*., 1996, Hill & Manley, 2009) and a first screening revealed that the selected alga is also a rich source of such compounds. The formation of low molecular weight halogenated metabolites is widely distributed in macro- and microalgae which contribute significantly to the atmospheric halocarbon budget (Sturges *et al*., 1992, Carpenter *et al*., 2003, Paul & Pohnert, 2011). Local maxima of volatile halogenated metabolites are often observed in coastal regions but the function of these metabolites is poorly understood. Here we directly link the halocarbon chemistry of microalgae to an allelopathic activity by establishing that the novel natural product cyanogen bromide BrCN is highly inhibitory against competitors. This metabolite is released during a short period after the onset of light in quantities sufficient to kill or inhibit the growth of competing microalgae.

## Methods

### Algal strains and culture conditions

We isolated diatom strains from the “Rammekenshoek” intertidal mudflat in the Westerschelde Estuary, The Netherlands (51°26'50" N, 3°38'38" E) on March 03, 2009. Cultures and permanent slides of the cultures used for the experiments are kept in the Laboratory of Protozoology and Aquatic Ecology, Ghent University, Belgium. We established clonal cultures as described in Chepurnov *et al* (2002). We prepared culture medium by filtering and autoclaving North Sea seawater enriched with f/2 nutrients (Guillard, 1975). We maintained clonal cultures in a climate room at 19 ± 1 °C and illuminated by cool-white fluorescent lamps at a rate of 50 μmol photons m⁻² s⁻¹ with a light/dark cycle of 12/12 hours. Iodine enriched culture medium was composed of ESAW artificial seawater (Berges *et al*., 2001) supplemented with 45 μM KI. Culture medium enriched with 13C was based on ESAW artificial seawater with 2.07 mM NaH¹³CO₃.
Bi-algal culture experiments

We examined the growth interactions between *Nitzschia cf. pellucida* and 3 benthic diatom species belonging to various genera (*Navicula arenaria* Donkin, *Cylindrotheca closterium* (Ehrenberg) Reiman & Lewin, and *Entomoneis paludosa* (W. Smith) Reimer) using bialgal cultures. We performed growth experiments in polystyrene 24-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany) containing 2.0 mL f/2 culture medium. We inoculated each species at a cell density of ~ 5,000 cells mL\(^{-1}\) resulting in an initial density of ~ 50 cells mm\(^{-2}\). We included monoculture treatments (inoculated at 10,000 cells mL\(^{-1}\)) as a control. We harvested cells for inoculations from monoclonal, exponentially growing cultures. We replicated each treatment four times. We daily renewed the culture medium (1.0 mL of double strength f/2 medium) to avoid nutrient limitation and hence resource competition for nutrients. We daily monitored the cell density of each replicate using an inverted microscope (Axiovert 135 Zeiss microscope, Jena, Germany) by counting a minimum of 300 cells per replicate.

Effects of spent medium

We prepared *N. cf. pellucida* spent medium by filtering exponentially growing cultures (200,000 – 250,000 cells mL\(^{-1}\)) on GF/F filters (Whatman, Maidstone, UK) and subsequently on 0.2 \(\mu\)m membrane filters (Acrodisc, Pall Life Sciences, Ann Arbor, MI, USA). We enriched this filtered spent medium with f/2 nutrients and then applied it to cells of *C. closterium*, *Staurovella* sp. and *E. paludosa*. In parallel, we cultured cells of these species with f/2 enriched seawater as a control. We monitored the effect of *N. cf. pellucida* spent medium by measuring the biomass and photosynthetic efficiency using Pulse-amplitude-modulated (PAM) fluorescence.

We used PAM fluorescence (MAXI Imaging PAM fluorometer, Walz, Germany) to determine the maximum quantum yield of photosystem II (PSII), which is frequently applied as a proxy for photosynthetic efficiency (Kromkamp *et al.*, 1998). We determined photosynthetic efficiency as \(\frac{F_v}{F_m}\), where \(F_v = F_m - F_o\). \(F_m\) is the maximum fluorescence emission level in the dark measured with a saturating pulse of light (emission peak at 450 nm, 2700 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\))
5s, 800 ms). We used the initial fluorescence $F_0$ as a proxy for biomass (Honeywill et al., 2002).

**Liquid-Liquid extraction of allelopathic compounds and GC-MS analysis**

We filtered 150 mL *N. cf. pellucida* spent medium (GF/F, Whatman, Maidstone, England) and extracted it 3 times with 50 mL ethylacetate. We dried the extract with anhydrous sodium sulfate and concentrated it at reduced pressure. We performed GC-EI-MS measurements of the concentrated extracts with a Waters GCT premier (Waters, Manchester, UK) time of flight mass spectrometer (MS) coupled to an Agilent 6890N gas chromatograph (GC) equipped with a DB-5ms column (30 m x 0.25 mm internal diameter, 0.25 μm film thickness and 10 m Duraguard pre-column, Agilent, Waldbronn, Germany). The carrier gas was Helium 5.0 with a constant gas flow of 1.0 mL min$^{-1}$. The source temperature was at 300 °C with an electron energy of 70 eV. The column was held at 40 °C for 2 min, heated up from 40 °C to 150 °C with 5 °C min$^{-1}$, from 150 °C to 280 °C with a rate of 20 °C min$^{-1}$ and held for 4.5 min. The samples were injected in splitless mode.

**Volatile organic compounds**

We used solid phase microextraction (SPME, Carboxen/Polydimethylsiloxane, Supelco, Bellefonte, PA) to measure the volatile compounds emitted by *N. cf. pellucida* cultures. We exposed the SPME fiber for 30 min to the headspace of 82 mL magnetically stirred filtrate (GF/F filters) of *N. cf. pellucida*. We used CDCl$_3$ (Eurisotop, Gif-sur-Yvette, France) (at 1.24 μM) as an internal standard to enable quantification. We analyzed the extracted compounds using the GC-MS described above. We calibrated the SPME extracting by measuring a dilution series of commercially available BrCN (Sigma Aldrich, Germany) with CDCl$_3$ as an internal standard. We determined BrCN concentrations by calculating the GC peak area using standard program peak detection. We normalized the BrCN peak area to the CDCl$_3$ peak area and used the calibration curve to calculate the BrCN concentrations in the *N. cf. pellucida* cultures.
Bioassays

We used bioassays to detect the presence of allelochemicals in *N. cf. pellucida* spent medium and used *E. paludosa* as the susceptible strain. We inoculated cells of exponentially growing *E. paludosa* in *N. cf. pellucida* spent medium at a final density of 2,000 cells mL\(^{-1}\). After two hours exposure to spent medium, we checked for the occurrence of resting cell formation and cell lysis using an inverted microscope (counting min 300 cells per replicate).

To check the occurrence of allelochemicals in the ethylacetate extracts, we concentrated the extracts using reduced pressure and finally dried the extract under a nitrogen enriched atmosphere. We dissolved the residue in acetone and added to an *E. paludosa* culture with 2,000 cells mL\(^{-1}\) at a final concentration of 1% acetone. This acetone concentration did not affect cell integrity itself within the timeframe of the bioassay.

We tested the toxicity of nine halogenated compounds on the diatom *E. paludosa* at concentrations of 0.1, 1, 5 and 10 µM (Fig. 4). We first dissolved the halogenated compounds in acetone and added to an *E. paludosa* culture with 2,103 cells mL\(^{-1}\) at a final concentration of 1% acetone. We microscopically checked for lysed cells, resting cells, and healthy cells 3h after application. We used the same approach to check the toxicity of NaCN on *E. paludosa* cells at 2, 10, 20 and 40 µM NaCN.

Synthesis of cyanogen iodide

We performed ICN synthesis as previously described (Bak, 1952). We dissolved 0.25 mmol NaCN (Sigma Aldrich) in 0.5 mL water and cooled it to 0 °C. We gradually added 0.25 mmol iodine (Fluka, Germany), waiting until the last portion has reacted. We extracted the watery solution 3 times with diethyl ether, dried the ether extract with sodium sulfate and removed it by a stream of argon. ICN was received as colourless crystals.

Catalase experiment

We assessed the effect of the H\(_2\)O\(_2\)-decomposing enzyme catalase (600 units bovine liver catalase mL\(^{-1}\) dissolved in water, Sigma Aldrich) on the toxicity of *N. cf. pellucida* cultures. We added catalase to the *N. cf. pellucida* cultures one hour before the onset of light and we tested the presence of allelochemicals three hours after the onset of light using the
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*E. paludosa* bioassay. We included a control treatment in which we stirred *N. cf. pellucida* cultures (analogous to the catalase treatment) one hour before the onset of light. We replicated treatments 4 times.

**Phenol red assay**

We used the bromination of phenol red (phenolsulfonphthalein) into brominated phenol blue (3',3'',5',5''-tetrabromophenolsulfonphthalein) as an indicator for halogenating activity (Hill & Manley, 2009). The conversion of phenol red into brominated phenol blue indicates the involvement of haloperoxidase enzymes. These enzymes catalyze the oxidation of halide ions to hypohalous acid by H₂O₂. Hypohalous acid (or a similar oxidized intermediate) can then react with organic substrates that are susceptible to electrophilic halogenation (Butler & Sandy, 2009). We added phenol red (30 μM final concentration) to *N. cf. pellucida* cultures (200,000 – 250,000 cells mL⁻¹) three hours after daybreak. Two hours later, we measured phenol red and brominated phenol blue spectrophotometrically at 433 nm and 592 nm respectively (Hill & Manley, 2009). Prior to the measurements, we removed cells by filtering on a 0.2 μm filter and adjusted the pH to 6.5 with acetic acid.

**Results**

**Allelopathic effects of Nitzschia cf. pellucida**

Experiments with co-cultures of biofilm forming diatom species revealed that the diatom *N. cf. pellucida* (Fig. 1a) exerted strong allelopathic effects on other diatom species. We observed that the naturally co-occurring diatoms *N. arenaria* (Fig. 1d), *C. closterium* and *E. paludosa* (Fig. 1b) were inhibited and killed after 24h exposure to relatively low cell densities (7,000-10,000 cells mL⁻¹) of *N. cf. pellucida*. Another diatom, *Stauronella* sp. (Fig. 1c) was more resistant but was killed within 24h when exposed to circa 80,000 cells mL⁻¹ of *N. cf. pellucida*. The mechanism of inhibition appeared to be the same for all species investigated: exposure to *N. cf. pellucida* cells resulted in loss of pigmentation, shrivelling of chloroplasts and finally cell death (Fig. 1).
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**Figure 1.** Antagonistic effects of *N. cf. pellucida* on a naturally co-occurring diatom species. **a:** *Nitzschia* cf. *pellucida*, healthy cell, cell after 10 min exposure to *N. cf. pellucida* cells, dead cell after 120 min exposure. **b:** *E. paludosa*, healthy cell, cell after 30 h exposure to *N. cf. pellucida* cells, cell after 48 h exposure. **c:** *Stauronella* sp., healthy cell, cell after 30 h exposure to *N. cf. pellucida* cells, cell after 48 h exposure. **d:** *Navicula arenaria*, healthy cell, cell after 30 h exposure to *N. cf. pellucida* cells, cell after 48 h exposure. Scale bar = 20 μm.

Application of nutrient-enriched spent *N. cf. pellucida* culture medium on cells of different diatom species induced a collapse of photosynthetic efficiency (Fig. 2) and massive cell lysis within 2h for *E. paludosa* (Fig. 3) and *C. closterium*. The diatom *Stauronella* sp. was again more resistant and was able to maintain its photosynthetic efficiency, but its growth was suppressed for two days (measured as the initial fluorescence, F₀, a proxy for algal biomass) after which cell growth at rates similar to the control was restored.
An additional response was detected in *E. paludosa* since *N. cf. pellucida* spent medium induced *E. paludosa* cells to lose motility and cells displayed a strong condensation of the protoplast within 10 min after exposure (Fig. 1b). Prolonged exposure to the *N. cf. pellucida* medium caused massive cell lysis within 2-3h (Fig. 3). As this species is highly sensitive towards *N. cf. pellucida*, we selected it as a model for further bio-assay experiments.

The allelochemical potential of *N. cf. pellucida* varied dramatically with time of day. Using a bioassay with *E. paludosa* and cell free spent medium from *N. cf. pellucida* cultures sampled in 1h to 1h30 time intervals, we showed that allelopathic activity is highest between two to four hours after daybreak when application of spent medium resulted in nearly complete eradication of *E. paludosa* cells (Fig. 3). Five hours after the onset of light, the activity diminished and the effect was reduced to the induction of protoplast shrinkage in >90 % of *E. paludosa* cells (Fig. 3). Six hours after daybreak, most of the cells showed no effect.
of spent medium, with > 50 % healthy cells, increasing to nearly 100 %
towards the end of the night. This striking pattern suggests that labile,
reactive or volatile metabolites are responsible for the allelopathic
activity. Further studies therefore focused on the characterization of
metabolites present 3h after the onset of light when toxicity was
maximal.

Figure 3. Time dependent allelochemical potential of *N. cf. pellucida* throughout the
day. Graph shows the effect of *N. cf. pellucida* cell free spent medium harvested at
different time points on *E. paludosa* cells. We measured the effects 2h after exposure
to *N. cf. pellucida* filtrate. (Means ± S.D.)

**Extraction and structure elucidation of *N. cf. pellucida* allelochemicals**

We performed GC-MS analyses of ethylacetate extracts (EAe) of cell-free
spent culture medium and of volatile organic compounds collected by
headspace solid phase microextraction (HS-SPME) and revealed the
occurrence of 18 different brominated and iodinated volatiles in *N. cf. pellucida* cultures. These compounds were a mixture of methylhalogens (CH$_3$Br, CH$_3$I), dihalomethanes (CH$_2$Br$_2$, CH$_2$I$_2$, CH$_2$ClI*, CH$_3$BrI*) as well as trihalomethanes (CHBr$_3$, CHI$_3$, CHBr$_2$I*, CHBr$_2$Cl*, CHClI$_3$*) (EAe and SPME), 1-iodopropane (EAe), di and tri-halogenated acetaldehydes (dibromoacetaldehyde*, bromochloroacetaldehyde*, chlorodibromo-acetaldehyde*, EAe), and 1,2-dichloroethane(E Ae). We identified these compounds based on their retention time and mass spectra using GC-MS and, if not indicated otherwise, compared with commercially available or synthetic (Bak, 1952) standards (* = identified only by mass spectrometry and retention time). In addition to these metabolites that are known from marine algae (Paul & Pohnert, 2011), we could also detect the volatile and highly toxic cyanogen bromide, BrCN using SPME. A commercially available synthetic standard of this novel natural product provided material for the confirmation of the structure, quantification and a dose response assessment in bioassays with *E. paludosa*.

To check if these halocarbons caused the observed allelopathic interactions, we assessed the toxicity of different concentrations of the nine most abundant halogenated compounds and NaCN on the diatom *E. paludosa* (Fig 4). In these bioassays, cyanogen bromide turned out to be by far the most toxic halogenated metabolite produced by the algae. The minimal lethal concentration was 2 µM (causing 96% of the *E. paludosa* cells to die within 3h). BrCN is by far more potent compared to NaCN, which is active only in concentrations above 40 µM. BrCN concentrations in the *Nitzschia* cultures (320,000 cells mL$^{-1}$) (determined by SPME GC-MS with CDCI$_3$ as an internal standard) reached on average 5.18 µM (± 3.10; n=4). The concentration of BrCN can thus fully explain the lethal effect of the spent *N. cf. pellucida* medium. No other tested halogenated metabolites were active in the concentration range reached in cultures. Cells of *N. cf. pellucida* are more resistant to BrCN and could cope with concentrations of up to 16 µM with minor or no growth reduction, and only displayed a reduced growth and photosynthetic efficiency at 32 µM BrCN (Fig. 5).
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![Graph showing dose-response curve for E. paludosa for 9 halogenated compounds detected in N. cf. pellucida cultures. The grey square represents the average BrCN concentration in N. cf. pellucida cultures (three hours after onset of light) and the average response of E. paludosa to the N. cf. pellucida filtrates. (Means ± S.D.)](image)

Figure 4. Dose-response curve for E. paludosa for 9 halogenated compounds detected in N. cf. pellucida cultures. The grey square represents the average BrCN concentration in N. cf. pellucida cultures (three hours after onset of light) and the average response of E. paludosa to the N. cf. pellucida filtrates. (Means ± S.D.)

![Graph showing effect of BrCN on (A) growth (measured as F0) and (B) the photosynthetic efficiency of N. cf. pellucida. (Means ± S.D.)](image)

Figure 5. Effect of BrCN on (A) growth (measured as F0) and (B) the photosynthetic efficiency of N. cf. pellucida. (Means ± S.D.)

To further validate that BrCN is the major metabolite responsible for the allelopathic activity, we deprived N. cf. pellucida cultures of bromide and iodide. Omitting these halogens from the culture medium almost completely eliminated the allelochemical activity (Fig. 6). When we altered the I⁻ : Br⁻ ratio in the culture medium from ca. 1:2,000 in natural seawater to 1:16, production of brominated hydrocarbons ceased and was replaced by the formation of iodinated compounds. Likewise, BrCN production was reduced and instead there was formation
of cyanogen iodide (ICN), a second new natural product. Spent medium of *N. cf. pellucida* grown at high I:Br ratios was even more toxic than spent medium derived from natural seawater and caused massive cell lysis of *E. paludosa* cells within 45 min after application (Fig. 6).

![Figure 6. Cell death in *E. paludosa* following exposure to filtrate of *N. cf. pellucida* grown in media with different I:Br ratios (1:16 and 1:2,000) and in absence of I and Br. (Means ± S.D.).](image)

**BrCN biosynthesis**

Since BrCN and ICN are not known as natural products and since their production evidently relies on the availability of external halides, we aimed to verify their biogenic origin by further addressing their biosynthesis. Therefore we incubated *N. cf. pellucida* cells in culture medium enriched with 13C labeled bicarbonate for 5 days. Analysis of the isotope distribution of the cyanides provided proof for a biosynthetic origin. BrCN and ICN exhibited a 13C : 12C ratio of 1 : 1.13 and 1 : 1.18 in BrCN and ICN respectively while the cyanides from cells in natural seawater exhibited the natural ratio of 1 : 0.012. (Fig 7).
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Figure 7. GC-EI-MS spectra of BrCN (A and C) and ICN (B and D). A and B were harvested from N. cf. pellucida cultures grown in natural seawater based culture medium and C and D from cultures grown in NaHCO$_3$ enriched medium.

Figure 8. Cell death in *E. paludosa* following exposure to filtrate of *N. cf. pellucida*, to filtrate and catalase, to filtrate from *N. cf. pellucida* cultures incubated with catalase (*Nitzschia + catalase*) and to a filtered seawater control. (Means ± S.D.)
Biosynthetic considerations of BrCN or ICN suggested the presence of an oxidized halogen species. Known enzymes which could be involved are H$_2$O$_2$ consuming haloperoxidases (HPO). To test if allelochemical production is linked with cellular H$_2$O$_2$ production, we assessed the effect of the H$_2$O$_2$-decomposing enzyme catalase incubated with *N. cf. pellucida* cultures. Catalase application indeed largely suppressed the allelochemical potential in the *N. cf. pellucida* cultures. The activity cannot be attributed to the catalase enzyme itself since it did not inhibit the allelopathic activity of spent medium in control experiments (Fig 8).

In a second assay for haloperoxidase activity we added phenol red (phenolsulfonphthalein) at 36 µM to *N. cf. pellucida* cultures and spectrophotometrically checked for conversion into brominated phenol blue (3',3",5',5"-tetrabromophenolsulfonphthalein). Halogenation of phenol red occurred shortly after daybreak and phenol red concentration decreased to 26.38 ± 2.26 (mean ± s.d., n=5) µM and 8.60 ± 1.86 (n=5) µM bromophenol blue was formed within three hours.

**Discussion**

In this study we show that the benthic diatom *Nitzschia cf. pellucida* produces allelochemicals that cause chloroplast bleaching and a reduced photosynthetic efficiency leading to growth inhibition and massive cell lysis in naturally co-occurring competing microalgae. The allelopathic compounds are effective with low threshold concentrations which is demonstrated by the fact that medium from cultures with relatively low cell densities (8,000 cells mL$^{-1}$) was active against competitors. The threshold concentrations relevant for cell-cell interactions in our experiments are probably overestimated given the recent finding (Jonsson *et al.*, 2009) that local concentrations of allelochemicals (near the chemical envelope of cells) can be orders of magnitude higher than those in the well mixed cell-free filtrates which we used in our experiments.

Our work adds to a very limited number of studies demonstrating the molecular basis for chemically mediated interactions between biofilm forming microalgae. Using a combination of chemical analyses and bioassays we identified the highly reactive metabolite BrCN as the
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causative agent of the observed activity. This compound has not been previously detected as a natural product. BrCN is highly toxic and has been applied as fumigant and pesticide and was even briefly used as a chemical weapon during World War I (Hosch, 2009). BrCN is currently used to fragment proteins by hydrolyzing peptide bonds at the C-terminus of methionine residues (Mortvedt et al., 1991). Besides this effective metabolite, N. cf. pellucida also produces a diverse mixture of iodo- and bromocarbons with comparatively lower allelopathic properties. The halomethanes, halogenated acetaldehydes and iodopropane detected have been reported previously from micro- and macroalgae (Giese et al., 1999, Kamenarska et al., 2007, Butler & Sandy, 2009). Cyanogen bromide is hydrolyzed by water to release hydrogen cyanide (HCN) but we can exclude that the allelopathic effects were caused by cyanide alone since NaCN caused only minor effects when applied to our bio-assay species E. paludosa. Concentrations of 40 μM NaCN (20 times higher than the active BrCN concentration) caused no short term (8 h) effect on E. paludosa cells, which is mild compared to the toxicity of BrCN. Since BrCN is not a common natural product we verified if it might result from abiotic transformations in the medium or if it is a true natural product biosynthesized by the alga. 13C labeled bicarbonate was incorporated into BrCN and ICN in high yields confirming unambiguously a biogenic origin.

Application of the H2O2-decomposing enzyme catalase suppressed the allelochemical potential in the N. cf. pellucida cultures which suggests that haloperoxidase (HPO) activity (Butler & Sandy, 2009) is involved in the BrCN synthesis. Furthermore, we observed a preference for iodide over bromide incorporation, which corresponds with the halide selectivity for haloperoxidases (Verhaeghe et al., 2008). Also the absence of CICN in Br and I depleted cultures matches with the halide preference of algal HPO. Lastly, the conversion of phenol red into brominated phenol blue in the Nitzschia cultures points to the involvement of haloperoxidase enzymes. These enzymes catalyze the oxidation of halide ions to hypohalous acid by H2O2. Hypohalous acid (or a similar oxidized intermediate) can then react with organic substrates that are susceptible to electrophilic halogenation (Butler & Sandy, 2009). We can not, however, conclude whether biogenic CN (or equivalent) reacts with “Br” from the haloperoxidase reaction or if the transformation of halomethanes or other precursors to BrCN is involved in the biosynthetic pathway. CN production is known from a broad range of organisms, including bacteria,
fungi, insects, algae, and plants, as a means to avoid predation or competition but the production and several pathways to this metabolite are described (Knowles & Bunch, 1986).

Haloperoxidase enzymes are distributed in marine organisms including Rhodophyta, Phaeophyta, Chlorophyta (Paul & Pohnert, 2011) and Bacillariophyta (Moore et al., 1996). An important function of HPO is to scavenge harmful $\text{H}_2\text{O}_2$ produced during photosynthesis, photorespiration and other metabolic processes (Manley, 2002). It has also been suggested that HPO of marine organisms are involved in defense mechanisms such as the mediation and prevention of bacterial biofilm formation, but evidence for the involved metabolites was not given till now (Brochardt et al., 2001, Cosse et al., 2009). Here we provide a link between HPO activity and allelopathic potential supporting an ecological role for this enzyme in diatoms.

It is interesting to note that we only detected BrCN during the morning hours in the culture and only if we quickly extracted this reactive metabolite. Given the methodological difficulties in detecting highly reactive metabolites, it would be worth verifying the potential of other microalgae to produce this reactive metabolite, which would most likely not have been picked up in other determinations of halogenated volatiles. The mechanism introduced here could thus have a broader occurrence.

The production of BrCN only within few hours after daybreak coincides with the time span of hydrogen peroxide production in algal cells due to the Mehler reaction (Collen et al., 1995). A short term release of a toxic metabolite to suppress growth of competitors might be a highly efficient allelopathic strategy. Like a “molecular toothbrush” BrCN could eliminate the surrounding flora daily after sunrise, leading to increased access to nutrients present in the environment and even elevated concentrations resulting from nutrients leaking from killed cells. The clean and nutrient-rich area could then be used by $N$. cf. *pellucida* for effective proliferation in the absence of toxins. This diatom species is more resistant to BrCN compared to its competitors (Fig. 5) but at elevated concentrations still sensitive to the toxin. Thus the short term toxin burst is an effective means of reducing the risk of auto-toxicity and represents a novel strategy for allelopathic interactions.

In this study we illustrate that a highly active simple metabolite from diatoms has the potential to promote daily cleaning events around a biofilm forming diatom. Our results provide a novel mechanism by which
The molecular toothbrush of a microalga diatoms can generate microscale chemical territoria in which competitors are deterred or killed. Obviously, such strategy contributes to complex micro-landscapes maintained by interacting species and may boost the small-scale patchy growth habits of biofilm forming species. Our results also suggest a potential link between the globally significant emissions of volatile halocarbons released by marine algae and allelopathic activity.

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Chapter 8

General Discussion

Diversity - Ecosystem Functioning and Organismal Traits

The main aim of this thesis was to gain a better understanding of the diversity and functioning of estuarine benthic diatom communities. The relationship between biodiversity and ecosystem functioning (BEF) has now been documented for a wide range of ecosystems. These studies generally found positive effects of diversity on different measures of ecosystem functioning (reviewed by Balvanera et al., 2006, Worm et al., 2006, Stachowicz et al., 2007, Cadotte et al., 2008, Cardinale et al., 2011). Many studies suggest that organismal trait differentiation is required for positive diversity effects, but actual information on trait differentiation is mostly lacking (Hillebrand & Matthiessen, 2009, Reiss et al., 2009). These organismal traits govern energy and material flows and may also mediate environmental conditions such as disturbance and nutrient availability.

Cryptic diversity and ecophysiological trait variation

An even more fundamental requirement to gain insights in BEF relationships is a solid knowledge of the diversity before we can begin to assign organismal trait characteristics to species. Assessing microalgal diversity has traditionally been based on the morphology of species, but species delineation and hence diversity measures based on morphology can be deceiving. Many recent studies using multiple approaches including morphology, molecular data and breeding experiments to assess species boundaries have shown high levels of cryptic and pseudocryptic variation in microalgae (Saez et al., 2003, Beszteri et al., 2005, Slapeta et al.)
al., 2006, Evans et al., 2007) and therefore suggest that diversity has been severely underestimated (Mann, 1999). In this thesis I add two more examples (the dominant epipelic diatom species Navicula phyllepta (chapter 2) and Cylindrotheca closterium, chapter 3) to the expanding list of (pseudo)cryptic species. More importantly, I show how closely related and morphologically similar lineages in both taxa differ in their ecophysiological traits (salinity and thermal niche respectively). Our results show that these microbial marine species are not the ecological generalists we once thought they were, but consist of different lineages with distinct ecological preferences. These insights are not only important when assessing effects of diversity on ecosystem function, but also help understanding the mechanisms that promote cryptic species coexistence.

It is likely that many other currently recognized estuarine benthic diatom species are actually species complexes, consisting of several closely related lineages with different ecological preferences. For example, several common estuarine diatoms are known for their presence along broad estuarine gradients (Krammer & Lange-Bertalot, 1986-1991) and it is unclear whether these morphospecies represent cryptic species with complementary distributional ranges and physiological properties or single species with broad ecological tolerances. Navicula gregaria for instance, another dominant benthic diatom in many estuaries, seems to consist of different genetic lineages; Sabbe (1997) and Cox (1987) reported the occurrence of two different morphological groups within N. gregaria which display distinct ecological preferences, and experiments showed that different N. gregaria genotypes differ in their salt tolerance (Vaneislander, 2004).

Organismal Traits and Disturbance

Species' traits are not only important for species coexistence and biodiversity-ecosystem function relations (see below), they may also determine how species can cope with disturbance. Based on an extensive dataset of benthic diatom assemblages covering a wide range of hydrodynamic disturbance, we showed that growth form and cell size are important traits to predict the effect of disturbance on species richness (chapter 4). Our results are consistent with the intermediate disturbance hypothesis which implicitly assumes that there is a trade-off in functional traits determining competitive ability and disturbance tolerance (Haddad
et al., 2008). These trade-offs lead to maximal diversity when competitive and disturbance tolerant species are able to coexist (Haddad et al., 2008).

**Diversity-Ecosystem Functioning in Microalgae Communities**

Field studies on benthic and planktonic microalgae suggest that diversity can enhance ecosystem functions such as resource use efficiency and productivity (Forster et al., 2006, Ptacnik et al., 2008). Yet, as is true for most other BEF studies, the underlying mechanisms are unclear. By experimentally assembling diatom communities with decreasing diversity, we demonstrated a highly positive biodiversity effect on biomass production (chapter 5). Based on the additive partition methods designed by Loreau & Hector (2001), we inferred that complementarity effects (sensu Loreau & Hector (2001), thus incorporating both niche complementarity and facilitation) were responsible for positive diversity effects. However, these mathematically derived measures of complementarity do not fully correspond with the ecological meaning of niche complementarity and facilitation (Cardinale et al., 2011). Several studies have shown that complementarity effects (sensu Loreau & Hector 2001) can be the balance of both negative and positive interactions between species (Hooper & Dukes, 2004, Cardinale et al., 2007).

In our study, we showed that growth of a strain of *Cylindrotheca closterium* was significantly promoted by the availability of organic exudates of neighbouring diatom species, suggesting that mixotrophy may constitute an important mechanism mediating facilitative interactions between benthic diatom species. Again, different strains of *C. closterium* showed different capabilities to grow mixotrophically, confirming that there can be considerable cryptic variation in organismal traits in morphologically similar strains.

As yet, we do not have further evidence for other mechanisms of niche complementarity that caused the observed positive diversity effects in our experiments, but it is likely that complementary use of light and/or nutrients is also involved. Complementary nutrient uptake leading to higher resource uptake in more diverse communities has been shown for different macroalgae species which are able to use different nitrogen forms (Bracken & Stachowicz, 2006). In the case of benthic microalgae, Underwood and Provot (2000) showed that different microphytobenthic
species and strains display distinct preferences and tolerances for nitrate and ammonium. In addition, Nilsson and Sundback (1996) showed species specific differences in the ability to take up amino acids.

Complementarity in light use has recently been shown for phytoplankton communities. More diverse phytoplankton communities harbour on average a more diverse array of photosynthetically active pigments which results in a more efficient absorbance of the available light spectrum leading to a higher biomass accrual (Striebel et al., 2009). Moreover, planktonic cyanobacteria display complementary chromatic adaptation which allows them to tune their pigment composition to the prevailing light spectrum and adjust to the presence of other cyanobacteria with specific spectral light requirements (Stomp et al., 2004, Kehoe & Gutu, 2006).

In contrast to phytoplankton living in relatively deep mixed layers, microphytobenthos often faces a very steep gradient in light with cells in biofilms at a depth of 300-400 μm experiencing almost complete darkness (Paterson et al., 2003). In such conditions, light limiting and inhibiting conditions are separated by only a few hundred μm. Epipelic diatoms can cope with such harsh conditions by exhibiting vertical migration patterns which are synchronized with daily emersion (Paterson, 1989). Kromkamp et al. (1998) suggested that there is a further differentiation possible within these migration cycles. He suggested that species “micro-migrate” within the upper layers of the biofilm, with different species surfacing at different timings. This was confirmed in field experiments by Underwood et al. (2005) who demonstrated that different microphytobenthos species do display differentiation in migration timing and photophysiological adaptation to inhibiting light conditions (especially with respect to non photochemical quenching). The niche differentiation in light use could thus also contribute to the overall higher resource use efficiency and a higher productivity of more diverse biofilms.

**Evolutionary History and Diversity-Ecosystem Functioning**

The evolutionary history of species determines current species traits, therefore it has been proposed that evolutionary history influences biodiversity and ecosystem functioning relations (Kinzig et al., 2002, Loreau et al., 2002, Gravel et al., 2011). Gravel et al. (2011) showed that
bacterial communities of experimentally evolved specialists have a stronger BEF relation than bacterial communities of experimentally evolved generalists. Maherali and Klironomos (2007) showed that phylogenetically more diverse communities of mycorrhizal fungi caused a higher associated plant productivity. They reasoned that there is a lot of functional redundancy of mycorrhizal species within families, and thus that traits are conserved within families, corresponding with the idea of phylogenetic niche conservatism. To which extent this phylogenetic niche conservatism applies to higher clades of microalgae is currently unknown, but we show that, at least for climatic niches, niche conservatism is weak within the diatom genus *Cylindrotheca* (chapter 3). To which extent this fast adaptive nature is specific for *Cylindrotheca* or if it can be broadened to marine microalgae is open for further discussion.

**Chemically Mediated Biotic Interactions**

In addition to these examples of niche differentiation leading to higher resource efficiency use through complementary resource use, there is a growing awareness that biotic interactions mediated by chemical cues can also strongly influence ecosystem function (Hay, 2009). Microalgae produce and exude a wide array of different molecules that are involved in selection of mates (Tsuchikane et al., 2008), defence against grazing (Miralto et al., 1999), repelling competitors (Gross, 2003), etc. These secondary metabolites are supposed to be omnipresent, but their identity is often unknown (Hay, 2009). These chemical cues have been described as the “language of life in the sea” and a better understanding of this language will evidently increase our understanding of biotic interactions and their importance for ecosystem functioning (Hay, 2009). The combination of chemical characterization methods and advanced ecological assays has only recently led to the identification of a relatively small set of chemical cues that affect the behaviour and/or physiology of marine microorganisms (Pohnert et al., 2007, Hay; 2009, Poulson et al., 2009), but their precise effects on ecosystem functioning and community structure are still virtually unknown.

How chemical cues affect the development and spatial organization of biofilms is poorly understood. Phototrophic biofilms, dominated by eukaryotic microalgae, are known to display complex microscale patchiness which cannot readily be explained by abiotic heterogeneity or bioturbation by grazers. It has therefore been
hypothesized that allelopathic interactions between biofilm eukaryotic microalgae may be invoked to explain this micro-scale patchiness (Saburova et al., 1995). In Chapter 6, I show that three different benthic diatom species produce allelopathic substances in a density-dependent way. These findings suggest that allelopathic interactions might be more common in benthic microalgal communities than previously thought. Furthermore, I demonstrate the physiological and molecular basis of allelopathic interactions caused by *Nitzschia* cf. *pellucida*. I show that this marine benthic diatom exudes a diverse mixture of volatile halogenated hydrocarbons including the highly toxic and new natural product cyanogen bromide (BrCN). Biosynthesis of the toxin is light-dependent, with a short burst of production shortly after the onset of light. Inhibiting cellular H$_2$O$_2$ production prevented the formation of allelochemicals, strongly suggesting that haloperoxidase enzyme activity (which is H$_2$O$_2$ dependent) is involved in BrCN synthesis. These antagonistic interactions have the potential to generate micro-scale chemical landscapes which may result in the observed small-scale patchy growth habits of biofilm forming species.

This work adds to a very limited number of studies demonstrating the molecular basis for chemically mediated interactions between microalgae. Production of volatile halogenated metabolites is widespread among micro- and macroalgae and contributes significantly to the global atmospheric halocarbon budget, but their biological role has always been poorly understood (Sturges et al., 1992, Carpenter & Liss, 2000, Salawitch, 2006, Paul & Pohnert, 2011). Furthermore, our results suggest a potential function for haloperoxidase enzymes, which are present in marine red, green and brown algae, diatoms, fungi and bacteria, but whose role to date remained elusive. Several studies have demonstrated allelopathic activity in algae possessing these enzymes, but a direct link between these enzymes and allelopathic activity has never been suggested. In addition, production of the toxin BrCN is coupled with photosynthetic activity, and raises interesting questions regarding photosynthesis related H$_2$O$_2$ production (e.g. in the Mehler reaction, Colen et al., 1995), haloperoxidase activity and allelochemical production.

Many other allelopathic interactions have been described (reviewed by Gross, 2003, Legrand et al., 2003), but only a few studies actually show the chemical nature of the interaction (Leao et al., 2010). Chemical cues may also mediate access to nutrients and thus influence
resource use efficiency. For instance, when it comes to phosphate acquisition, certain cyanobacteria have a cunning plan. Bar-Yosef et al. (2010) showed that *Aphanizomenon ovalisporum* produces the cyanotoxin cylindrospermopsin which causes the production of extracellular phosphatases in other phytoplankton species. These enzymes break down external phosphate esters (which can not be readily assimilated) with the production of inorganic phosphate which can then be (partly) used by *Aphanizomenon*. Other chemical cues that govern resource use efficiency are exchanged between certain bacteria and phytoplankton species. Certain symbiotic bacteria can help microalgae by increasing the solubility of boron and iron by producing non-specific boron and iron binding siderophores which can be shared with microalgae (Amin et al., 2007, Amin et al., 2009). Bacteria may also enhance microalgal growth by suppling microalgae with essential compounds such as cobalamin (vitamin $B_{12}$) (Croft et al., 2005, King et al., 2011).

**Conclusion**

Overall, our results contribute to the understanding of the species composition and functional organization of complex phototrophic biofilms and its consequences for ecosystem functioning. We suggest that cryptic diversity is widespread among estuarine diatoms and that this diversity is linked with ecophysiological differentiation and habitat partitioning by closely related species. Over large environmental gradients it appears that lineages behave in a conservative fashion, in the sense that they are limited in their distribution to suitable hydrodynamical conditions. At the local scale, strong positive and negative interactions (facilitation, allelopathy) appear to be important in these diatom communities.
Perspectives

The results in this thesis invite further research in several research areas. I highlight some of the specific topics that, in my opinion, deserve further research.

Next Generation BEF Experiments

Our laboratory BEF experiment yielded insights into the potential mechanisms responsible for enhanced performance of more speciose biofilms. Yet, this experiment largely ignored the natural complexity, thus its applicability to real natural systems is unclear. Future experiments should include more environmental realism such as the inclusion of natural environmental variation and trophic complexity and should explore the impact of diversity loss at greater spatial and temporal scales. The effect size of diversity should be compared with the effect sizes of environmental variation and heterogeneity (see also Cardinale et al., 2011).

Next to these larger scale experiments, there is a long-lasting need to gain more insights into the mechanisms and functional traits responsible for BEF relations. Ideally, BEF experimental designs could combine measurements of multiple biogeochemical processes, a metabolomics approach that scans the extracellular chemical cues, and a monitoring of key cell functions by profiling the expression of selected genes. Or more simply: linking the ‘omics’ to community ecology to better understand how organismal traits affect species interactions and community functioning.

Adaptation in Marine Microalgae

On the basis of the results presented in chapter 3 in which we focused on the thermal niche of closely related lineages within the marine diatom genus *Cylindrotheca*, we can draw some further questions that merit study: How fast can a strain adapt to another temperature regime? Which genes are involved in temperature adaptation? Can this adaptation potentially influence speciation? Is ecological speciation important for diversification in marine phytoplankton? Is the negative correlation between cold and heat tolerance caused by antagonistic
pleiotropy or mutation accumulation? Part of these questions could be resolved by conducting evolution experiments with e.g. cold adapted strains and let them adapt gradually to sublethal temperatures (similar to Bell & Gonzalez, 2009). Differences in gene expression between adapted and ancestral strains could then be assessed to gain more knowledge in the genes responsible for thermal adaptation.

Ecological Importance of Microalgal Halocarbon Chemistry

Many marine micro- and macroalgae produce volatile halogenated metabolites, an emission which is of global significance, but why they do this is not understood (Paul & Pohnert, 2011). Several macroalgae such as the green alga *Ulva* sp. and the red algae *Gracilaria* sp. and *Corallina* sp. are known to possess haloperoxidases (Sheffield et al., 1995, Manley & Barbero, 2001, Ohsawa et al., 2001, Weinberger et al., 2007) and other reports have shown allelopathic interactions for these species (Jeong et al., 2000, Wang et al., 2009), but no reliable mechanistic link nor halogenated allelochemicals have been identified so far for these species. Next to these algae with known halogenating activities, several dinoflagellates produce unknown toxins that are associated with hydrogen peroxide and superoxide (Marshall et al., 2005). In at least one case, toxin production is H$_2$O$_2$ dependent (Kim et al., 1999) but the identity of the active compound remains unknown (Kim et al., 2002). An enhanced effort with the methods described in this thesis might yield new insights in the ecology of halocarbon chemistry.

Haloperoxidase enzymes also have the potential to disrupt bacterial quorum sensing (a cell-cell communication system that enables a single cell to sense the total cell number of its population, governed by the accumulation of signaling molecules). Quorum sensing (QS) disruption has already been shown for the brown algae *Laminaria digitata* (Brochardt et al., 2001). It might be interesting to test if the halogenating enzymes in the diatom *Nitzschia* cf. *pellucida* are also able to enzymatically alter or degrade bacterial QS signals such as N-acylhomoserine lactone (AHL) which can be easily checked using standard bacterial bioassays (Dobretsov et al., 2009).
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General Discussion

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Summary

Humanity has become a major biogeochemical force and human domination of the planet has caused massive destruction and fragmentation of natural habitats, eutrophication, climate change, acidification, etc which caused a dramatic biodiversity loss. This biodiversity crisis raised concern about the consequences of biodiversity loss on ecosystem properties and the goods and services these ecosystems provide to humanity.

The main aim of this thesis was to gain a better understanding of the diversity and functioning of estuarine benthic microalgae communities. Estuarine ecosystems provide some essential ecosystem services to humans such as carbon fixation, sustaining fisheries, coastal protection and nitrogen cycling. Although these estuaries cover relatively small areas on a global scale, their importance for several biogeochemical processes is disproportionately large. We focused on benthic microalgae inhabiting estuarine sediments; these algae have many essential functions for estuarine and coastal ecosystems: e.g. they are the basis of the food chain for many coastal species, mediate nutrient fluxes and stabilize sediments.

A fundamental requirement to gain insights in biodiversity and ecosystem function relationships is a solid knowledge of the diversity. Assessing diversity is not as trivial as it may seem at first, especially in microbial communities. Traditionally, species have been defined based on morphological properties. A multitude of molecular studies however have demonstrated that morphologically defined species often consist of several genetically distinct lineages. Such genetically distinct but morphologically indistinguishable species are called “cryptic species”, whereas the term “pseudocryptic species” refers to the occurrence of morphologically similar species which at a closer look do exhibit subtle morphological differences between species. In most cases it is unknown whether (pseudo)cryptic species differ in their ecological preferences or whether they are functionally equivalent. This notion is not only important for questions relating diversity and ecosystem function, but also to understand mechanisms that promote cryptic species coexistence.

In Chapter 2, we used sequence data of 3 different genetic markers (ITS rDNA, the 18S rRNA gene and rbcL) together with cell
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Wall morphology to show that estuarine populations of the widespread and common benthic diatom *Navicula phylepta* Kütz. consist of pseudocryptic species. Moreover, we show that these pseudocryptic species differed in their tolerance to low salinities (<5 practical salinity units, psu), which was reflected by their different (but widely overlapping) distribution in the Westerschelde estuary (the Netherlands). Our results show that *N. phylepta* sensu lato comprises different species with specialized ecophysiological characteristics rather than generalists with a broad adaptability to different environmental conditions.

In Chapter 3 we further investigated the ecological divergence in closely related (pseudo)cryptic microalgae and assessed pattern in phylogenetic relatedness and ecological niche similarity. We explored if thermal niches can evolve fast on an evolutionary timescale resulting in regular switches between climates or if these niches are instead highly conserved. We composed a set of closely related strains of the globally distributed diatom genus *Cylindrotheca*. We collected strains from a wide range of marine habitats, from coastal plankton to sea ice and intertidal mudflats. We first inferred the evolutionary relationships of these strains using a multi-locus DNA dataset and obtained a well-resolved phylogeny. We then determined temperature preferences of closely related lineages in laboratory experiments. Combining the molecular phylogeny with the thermal niches of lineages revealed a very weak phylogenetic signal in thermal niche characteristics. This indicates that closely related species tend to differ more in thermal niche than expected by a random walk model. This seems to be caused by a combination of adaptive evolution and frequent shifts in environments in related lineages.

Species specific niches and functional traits are not only important for species coexistence and biodiversity-ecosystem function relations (see below), they may also determine how species can cope with disturbance. In Chapter 4, we examined the relations between the hydrodynamic disturbance, biomass, species diversity and functional group turnover in estuarine intertidal microphytobenthos. We used an extensive dataset of benthic diatom assemblages covering a wide range of hydrodynamic disturbance to show that growth form and cell size are important traits to predict the effect of disturbance on functional group species richness. Total microphytobenthos species richness displayed a unimodal relationship with hydrodynamic disturbance and standing stock
Summary

biomass. Our results are consistent with the intermediate disturbance hypothesis which implicitly assumes that there is a trade-off in functional traits determining competitive ability and disturbance tolerance. These trade-offs lead to maximal diversity when competitive and disturbance tolerant species are able to coexist.

In Chapter 5 we assessed the effects of species diversity on productivity of intertidal microphytobenthos. We used naturally co-occurring diatom species from intertidal mudflats to experimentally assemble communities with decreasing diversity. Our results demonstrate a highly positive biodiversity effect on production, with mixtures outperforming the most productive component species in more than half of the combinations. These strong positive diversity effects could largely be attributed to facilitation and complementarity effects. In addition, we show mechanistic evidence for facilitation which is partly responsible for enhanced production. We show that a strain of *Cylindrotheca closterium* has the ability to significantly increase its biomass production in response to substances leaked into the culture medium by other diatom species. In these conditions, the species drastically reduced its pigment concentration, which is typical for mixotrophic growth.

In Chapter 6, I show that, next to positive, facilitative interactions, direct negative interactions (allelopathy) can strongly influence species composition in microphytobenthos. I show that cell cultures of the marine benthic diatom *N. cf pellucida* exude metabolites with strong allelopathic effects. All nine competitor species tested were inhibited in their growth and some in their photosynthetic efficiency as well. In addition, I show the occurrence of reciprocal, density dependent allelopathic interactions between *N. pellucida* and two other marine benthic diatom species, *Entomoneis paludosa* and *Stauronella* sp. These results suggest that allelopathic interactions might be common in benthic microalgal communities and may explain small -scale spatial distribution patterns observed in nature.

In Chapter 7, I demonstrate the physiological and molecular basis of allelopathic interactions caused by *Nitzschia* cf. *pellucida*. I show this marine benthic diatom produces chemical cues that cause chloroplast bleaching and a reduced photosynthetic efficiency leading to growth inhibition and massive cell lysis in naturally co-occurring competing
Summary

Using headspace solid phase microextraction (HS-SPME) - GC-MS, I demonstrate that this diatom exudes a diverse mixture of volatile iodinated and brominated metabolites including the new natural products cyanogen bromide (BrCN) and cyanogen iodide (ICN) which exhibits pronounced allelopathic activity. Besides these effective metabolites, this diatom also produces a diverse mixture of mono-, di- and trihalomethanes, halogenated acetaldehydes and iodopropane with comparatively lower allelopathic properties. Production of these toxins is light-dependent with a short toxin burst after sunrise. This labile compound thus acts as a short-term signal, leading to daily “cleaning” events around the algae. We show that the allelopathic effects are \( \text{H}_2\text{O}_2 \) dependent and therefore link BrCN production to haloperoxidase activity. This novel strategy of chemical warfare is a highly effective means of biofilm control. Obviously, such strategy contributes to complex micro-landscapes maintained by interacting species and may boost the small-scale patchy growth habits of biofilm forming species. Our results also provide a potential explanation for the poorly understood role of volatile halocarbons from marine algae which contribute significantly to the atmospheric halocarbon budget.

Overall, our results contribute to the understanding of the species composition and functional organization of complex phototrophic biofilms and its consequences for ecosystem functioning. We suggest that cryptic diversity is widespread among estuarine diatoms and that this diversity is linked with ecophysiological differentiation and habitat partitioning by closely related species. Over large environmental gradients it appears that lineages behave in a conservative fashion, in the sense that they are limited in their distribution to suitable hydrodynamical conditions. At the local scale, strong positive and negative interactions (facilitation, allelopathy) seem to be important in these diatom communities.
Samenvatting

De menselijke overheersing van de planeet veroorzaakt een massale vernietiging en versnippering van natuurlijke habitats, eutrofiering, klimaatverandering, verzuring, etc die allen leiden tot een drastisch verlies aan biodiversiteit. Deze biodiversiteitscrisis leidde tot ongerustheid over de gevolgen van biodiversiteitverlies voor het functioneren van ecosystemen en de goederen en diensten deze ecosystemen leveren aan de mensheid.

Het belangrijkste doel van dit proefschrift was om een beter begrip van de biodiversiteit en het functioneren van estuarine benthische microalgen gemeenschappen te verkrijgen. Estuarine ecosystemen leveren een aantal essentiële ecosysteemdiensten zoals koolstoffixatie, het ondersteunen van de visserij en de bescherming van de kustgebieden. Ondanks dat deze estuaria slechts een relatief klein oppervlak innemen op wereldschaal, is het belang van estuaria voor verschillende biogeochemische processen onevenredig groot. Deze scriptie focust op estuarine benthische microalgen; deze algen hebben vele essentiële functies voor estuarine en mariene ecosystemen. Ze vormen de basis van de voedselketen voor vele kustsoorten, beïnvloeden nutriëntencycli en zorgen voor sediment stabilisatie.

Een fundamentele vereiste om inzicht te verkrijgen in de relatie tussen biodiversiteit en ecosysteemfuncties is een gedegen kennis van de diversiteit. Het schatten van de diversiteit van microbiële gemeenschappen is niet zo triviaal als het lijkt. Traditioneel zijn soorten gedefinieerd op basis van morfologische eigenschappen. Een veelheid van moleculaire studies hebben echter aangetoond dat morfologisch omschreven soorten echter vaak bestaan uit meerdere genetisch verschillende groepen. Dergelijke genetisch te onderscheiden, maar morfologisch niet te onderscheiden soorten worden 'cryptische soorten' genoemd, terwijl de term "pseudocryptische soorten" verwijst naar morfologisch verwante soorten die subtiele morfologische verschillen tonen. In de meeste gevallen is het onbekend of (pseudo)cryptische soorten verschillen in hun ecologische voorkeuren of dat ze functioneel gelijkwaardig zijn. Inzicht in de ecologische niche van nauw verwante (pseudo)cryptische soorten is niet alleen belangrijk voor vragen betreffende diversiteit en het functioneren van ecosystemen, maar ook
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voor een beter begrip van de mechanismen die samen voorkomen van cryptische soorten bevorderen.

In Hoofdstuk 2 gebruikten we DNA sequentie gegevens van 3 verschillende genetische merkers (ITS rDNA, het 18S rRNA-gen en rbcL) samen met morfologische kenmerken van de celwand om aan te tonen dat estuariene populaties van de wijdverspreide bentische diatomee *Navicula phyllepta* bestaan uit verschillende pseudocryptische soorten. Bovendien tonen we aan dat deze pseudocryptische soorten verschillen in hun tolerantie voor lage zoutconcentraties (<5 PSU), wat tot uiting kwam door hun verschillende (maar grotendeels overlappende) verspreiding in de Westerschelde (Nederland). Onze resultaten tonen aan dat *N. phyllepta* sensu lato bestaat uit verschillende soorten met specifieke ecofysiologische karakteristieken, het is dus geen generalistische soort met een breed aanpassingsvermogen aan verschillende milieumomstandigheden.

In Hoofdstuk 3 onderzochten we verder of er ecologische verschillen tussen de nauw verwante (pseudo)cryptische microalgen bestaan en onderzochten we de relatie tussen fylogenetische verwantschap en gelijkenis in ecologische niches. We onderzochten of temperatuursniches snel kunnen evolueren op een evolutionaire tijdschaal. We verzamelden hiervoor een reeks van nauw verwante stammen van het wereldwijd verspreide diatomeeëngeslacht *Cylindrotheca*. We verzamelden stammen uit een breed scala van mariene habitats, van kust plankton tot zee-ijs en intertidale slikken. We onderzochten de evolutionaire relaties van deze stammen met behulp van een multi-locus DNA-dataset en verkregen een goed ondersteunde fylogenetische boom. Daarna bepaalden we de temperatuurpreferenties van nauw verwante stammen in ecofysiologische experimenten. Het combineren van de moleculaire fylogenie met de temperatuursniche van deze stammen toonde aan dat er een zeer zwak fylogenetisch signaal in de thermische niche was. Dit geeft aan dat nauw verwante soorten de neiging hebben om meer in thermische niche af te wijken dan verwacht door een random walk model. Dit lijkt te worden veroorzaakt door een combinatie van adaptieve evolutie en frequente verschuivingen in de leefomgeving van deze stammen.

Soortsspecifieke niches en functionele kenmerken zijn niet alleen belangrijk voor soorten samenleven en biodiversiteit-ecosysteem functie relaties (zie hieronder), zij kunnen ook bepalen hoe soorten kunnen
Summary

omgaan met verstoringen. In Hoofdstuk 4 onderzochten we de relaties tussen hydrodynamische verstoring, biomassa, diversiteit en functionele kenmerken van estuariene bodembewonende microalgen. We gebruikten een uitgebreide dataset van bodembewonende diatomeeëngemeenschappen. We toonden aan dat het effect van verstoring op functionele groep soortenrijkdom deels kan verklaard worden door de groeiform en celgrootte van de bodembewonende microalgen. De totale soortenrijkdom vertoonde een unimodale relatie met hydrodynamische verstoring. Onze resultaten zijn in overeenstemming met de intermediate disturbance hypothese.

In Hoofdstuk 5 onderzochten we de effecten van de soortenrijkdom op de productiviteit van estuariene bodembewonende microalgen. We gebruikten diatomeeënsoorten die samen in intergetijde modderplaten voorkomen om experimentele gemeenschappen met afnemende diversiteit samen te stellen. Onze resultaten wezen op een positief effect van diversiteit op de biomassa productie, waarbij soortenmengsels meer biomassa produceerden dan de meest productieve soorten in monocultuur. Deze sterk positieve effecten kunnen grotendeels worden toegeschreven aan facilitatie en niche complementariteit. Bovendien toonden we een mechanisme voor facilitatie. We toonden aan dat een stam van de diatomeeënsoort Cylindrotheca closterium de potentie heeft om zijn groei aanzienlijk te versnellen door suikers op te nemen die door andere diatomeeënsoorten uitgescheiden werden.

In Hoofdstuk 6, toon ik aan dat, naast positieve, faciliterende interacties ook direct negatieve interacties (allelopathie) sterk de soortensamenstelling van bodembewonende microalgen kan beïnvloeden. Ik toon aan dat celculturen van de mariene benthische diatomee N. cf pellucida metabolioten met sterke allelopathic effecten uitscheiden. Alle geteste diatomeeënsoorten werden geremd in hun groei en stierven uiteindelijk na blootstelling aan N. cf pellucida. Daarnaast toon ik dat er wederkerig, dichtheidsafhankelijke allelopathische interacties bestaan tussen N. pellucida en twee andere mariene benthische diatomeeën soorten, Entomoneis paludosa en Stauronella sp. Deze resultaten suggereren dat allelopathic interacties algemener kunnen zijn dan gebruikelijk verwacht wordt en deze interacties helpen meer inzicht te verkrijgen in kleinschalige ruimtelijke variatie in het voorkomen van soorten.
In Hoofdstuk 7, toon ik de fysiologische en moleculaire basis van allelopathic interacties veroorzaakt door Nitzschia cf pellucida. Ik toon aan dat deze mariene benthische diatomee metabolieten uitscheidt die chloroplasten van concurrerende soorten afbleken wat leidt tot een verlaagde fotosynthetische efficiëntie en uiteindelijk massale celsterfte. Met behulp van vaste fase micro-extractie (SPME) – gaschromatografie gekoppeld aan een massaspectrometer (GC-MS), toon ik aan dat deze diatomeeën een diverse mix van vluchtige jodiumhoudende en gebromeerde metabolieten produceert, inclusief de nieuwe natuurlijke producten broomcyanide (BrCN) en iodiumcyanide (ICN). Naast deze extreme giftige metabolieten produceert deze diatomeeënsoort ook een diverse mix van mono- di- en trihalomethanen, gehalogeneerde acetaldehyde's en iodopropaan. De productie van deze gehalogeneerde producten is lichtafhankelijk, met een korte puls van BrCN kort na zonsopgang. Deze labiele verbinding werkt dus als een korte-termijn-sinaal, wat leidde tot de dagelijkse 'reiniging' rond de algen. We tonen aan dat de vorming van allelopathische stoffen waterstofperoxide (H₂O₂) afhankelijk is en linken dus BrCN productie aan haloperoxidase activiteit. Deze nieuwe strategie van chemische oorlogsvoering is een zeer effectief middel van biofilm controle. Dergelijke strategie draagt bij tot het ontstaan van complexe micro-landschappen door biotische interacties. Onze resultaten bieden ook een mogelijke verklaring voor weinig begrepen, maar globaal belangrijke uittuut van vluchtige gehalogeneerde koolwaterstoffen door mariene algen.

Als conclusie kunnen we stellen dat onze resultaten bijdragen tot een beter begrip van de soortensamenstelling en functionele organisatie van complexe fototrofe biofilms en de gevolgen daarvan voor het functioneren van deze ecosystemen. Wij suggereren dat cryptische diversiteit wijdverbreid is bij estuariene diatomeeën en dat deze diversiteit verbonden is met ecophysioogische differentiatie tussen nauw verwante soorten. Over grote milieu-gradiënten blijkt dat soorten zich op een conservatieve wijze gedragen, in de zin dat ze beperkt zijn in hun verspreidingsgebied worden om geschikte hydrodynamische omstandigheden. Op de lokale schaal zijn wellicht sterk positieve en negatieve interacties (facilitatie, allelopathie) belangrijk voor deze diatomeeën gemeenschappen.
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Curriculum vitae

Bart Vanelslander (°1981, Bruges, Belgium) graduated as a biologist at Ghent University in 2004. In 2005 he started a PhD in the Laboratory of Protistology and Aquatic Ecology in the Biology department of Ghent University. As a research assistant he organised and supervised many laboratory classes and field courses. During his PhD, Bart supervised many Bachelor and Master students during their thesis.

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Bart visited the Friedrich Schiller University of Jena (Germany) for two months where he worked at the chemical ecology lab of Prof. Georg Pohnert. Bart also visited the lab of Prof. Ulf Karsten (Rostock University, Germany) and the sediment ecology research group of Prof. David Paterson (St Andrews University, U.K.) for a study visit and a training course.

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