GHENT UNIVERSITY

FACULTY OF SCIENCES

Diatom-bacteria consortia from marine biofilms: assembly, interactions and adaptations

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Academic Year 2018-2019

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Dissertation submitted in fulfilment of the requirements for the degree of Doctor (Ph.D.) in Sciences: Marine Sciences (Ghent University)

LM-UGent LABORATORY of MICROBIOLOGY

PROTISTOLOGY AQUATIC ECOLOGY

SynBioC

I began to realize how important it was to be an enthusiast in life. He taught me that if you are interested in something, no matter what it is, go at it at full speed ahead. Embrace it with both arms, hug it, love it and above all become passionate about it. Lukewarm is no good. Hot is no good either. White hot and passionate is the only thing to be. - Roald Dahl, My Uncle Oswald

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This thesis was supported financially by the Fund for Scientific Research – Flanders (FWO-Flanders; 1115715N) and the BiodivERsA project BIO-Tide ((EU Horizon 2020 ERA-Net COFUND BiodivERsA)

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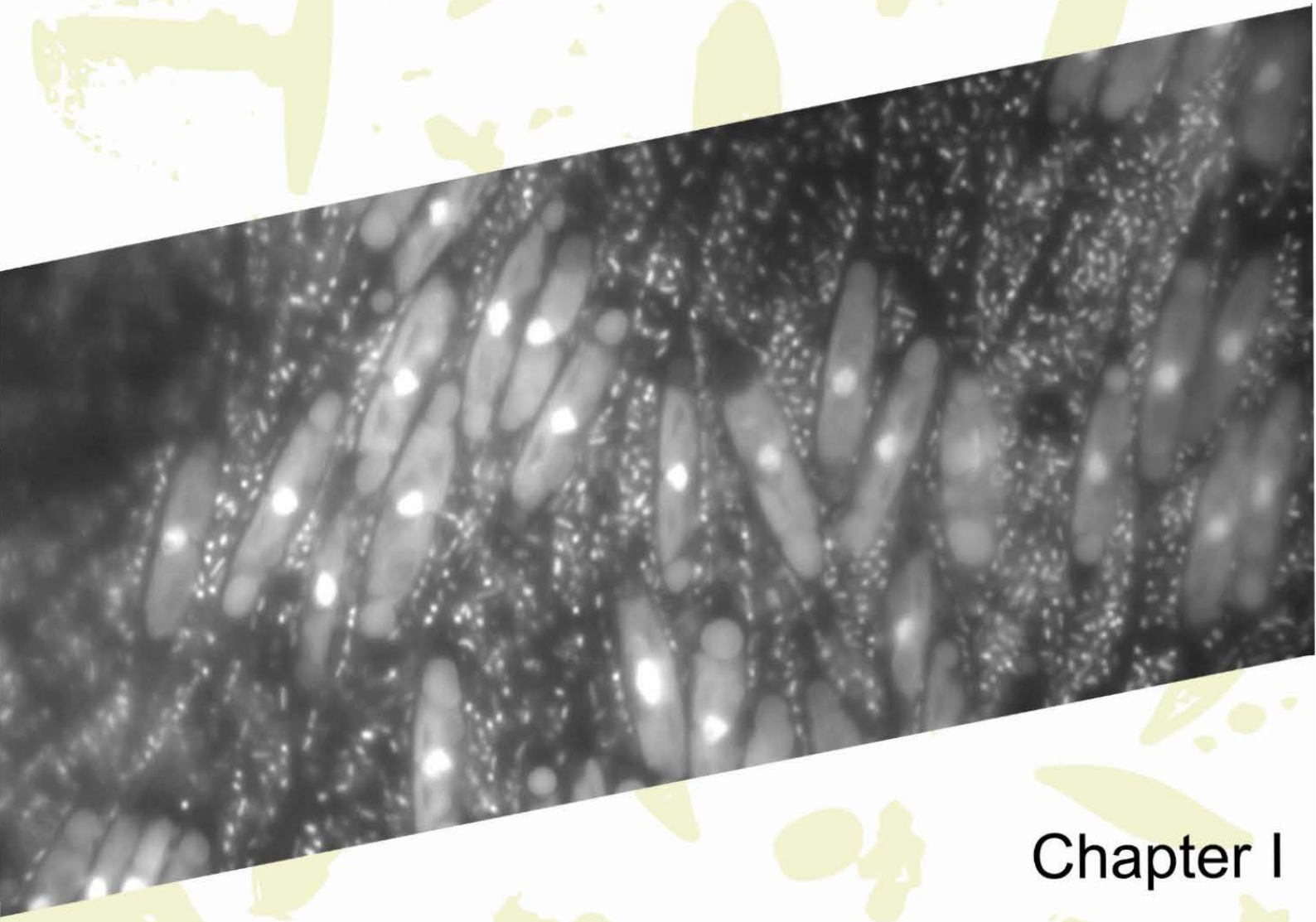

Chapter I

General introduction

All eukaryotic organisms grow, reproduce and interact in the presence of bacteria. It is in their best interest if they can manage their associated bacteria to their advantage during this time. Virtually all organisms have evolved mechanisms to do so, which has resulted in countless remarkable associations: termites have a unique gut microbiome capable of degrading complex plant material (Hongoh 2011), algae acquire essential vitamins from their associated bacteria (Croft et al. 2005), the right gut microbial metabolism improves the mental quality of life in humans (Valles-Colomer et al. 2019) and plants have growth-promoting bacteria covering their roots that simultaneously protect them from pathogens (Ahemad and Kibret 2014). Despite the importance of bacteria to our health and that of many other organisms, little is known about the mechanisms that shape the bacterial community during the long-term symbiosis with their host.

The assembly of a bacterial community into a host-associated microbiome is complex but can nonetheless be captured by four classes of processes: selection, drift, speciation and dispersal (Vellend 2010). Bacteria will be added to the regional bacterial pool via speciation and dispersal. This pool of bacteria will contain bacteria not able to colonize the host because their requirements are not compatible with the conditions or resources available on the host (Adair & Douglas 2017). In contrast, others will be particularly well-suited to live with the host (Brencic & Winans 2005; Powell et al. 2016). Selection (deterministic process as a result of fitness differences between species), drift (neutral processes due to ecological stochasticity) and dispersal will ultimately determine which bacteria from the regional bacterial pool will be associated with the host. These are the same processes acting on all bacterial communities, although their relative importance is scale and situation dependent (Nemergut et al. 2013). Unique to host associated bacterial communities is that the host not only serves as a habitat but can also actively interfere with the assembly process (Adair & Douglas 2017). The host can do this by secreting growth or reproductive stimulants for certain bacteria (Scheuring & Yu 2012), or by preventing the settlement of pathogens through the production of toxins. By expressing the influence of the regional bacterial pool, the host and the environment in terms of selection, drift, speciation and dispersal, we hope to gain a better understanding of the assembly process itself and the influences of each of these factors.

Once assembled, the association between host and bacteria can be ephemeral or the start of a long-lasting intimate relation between host and bacteria. The eco-evolutionary dynamics between hosts and bacteria can then range from a continuous ecological fitting process (Agosta & Klemens 2008), where suitable partners must be newly recruited in every generation, to a tight coevolutionary relationship between partners, where bacteria can ultimately become vertically transmitted organelles (McCutcheon & Moranc 2012). In both scenarios, interactions between host and bacteria have the potential to impact the fitness of the organisms involved, which can result in evolutionary changes in hosts and bacteria. These evolutionary changes can in turn affect the ecology of the organisms and how host and bacteria interact with one another, resulting in an eco-evolutionary feedback between hosts and bacteria (De Meester et al. 2018). The holobiont-hologenome concept has been proposed (Bordenstein & Theis 2015; Zilber-Rosenberg & Rosenberg 2008) to account for this complex eco-evolutionary dynamic by recognizing that the host is not an autonomous entity but rather presents an integrated and interacting network composed of the host and associated microbes. As a result, the host, together with its bacteria, can be a unit of selection in evolution (Theis et al. 2016).

The eco-evolutionary dynamic between hosts and their bacteria can result in interdependencies between host and bacteria (Kazamia et al. 2016). For instance, recurrent ecological associations between hosts and bacteria reduce selectional pressure on the partners to both maintain the genetic capacity to produce the same metabolites. Driven by an abiotic pressure for streamlined genomes (Black Queen Hypothesis; Morris et al. 2012) or by the presence of the other partner (Foraging-to-Farming hypothesis; Kazamia et al. 2016), this can lead to a loss of function and thus a dependency of one partner on the other arises (Kazamia et al. 2016). Such dependencies can evolve rapidly, as was for instance proven for a green microalgae, which developed a dependency for cobalamin within 500 generations (Helliwell et al. 2015).

The relation between a host and its bacteria tends to be asymmetrical as there generally is only one host interacting with a plethora of bacteria. By providing a fitness benefit to bacteria with favourable traits, the host can allow these bacteria to have a competitive advantage over the other bacteria. As a result, selection is expected to result in an enrichment of bacteria which tend to enhance the hosts' fitness (the

'Microbiome on a Leash concept'; Foster et al 2017). The asymmetrical relation is thus expected to lead to an eco-evolutionary dynamic which is particularly beneficial to the host, but not necessarily to the bacteria (Foster et al. 2017).

Despite these recent insights and new theories, our understanding on the eco-evolutionary dynamics of host-bacteria interactions remains limited (Limborg & Heeb 2018; Scheuring & Yu 2012). In a rapidly changing world, where we depend on hosts and their bacteria for trillions of euro per year for ecosystem services (Costanza et al. 2014) such as food production and wastewater treatment, a better insight in these dynamics is essential (Simon et al. 2019) and can result in marked socioeconomic benefits.

Part of the challenge in moving forward in our understanding of host-bacterial interactions lies in the development of novel model organisms. The success of data driven omics approaches have shifted the focus of this research away from cultivation-based approaches (Dittami et al. 2019). Nonetheless cultivation efforts must be maintained as much as possible to experimentally validate interaction hypotheses and investigate the mechanisms underlying the associations. Over the past decennia, a plethora of different organisms has been used to investigate interactions with bacteria, often with a different emphasis: sponge-bacteria interactions proved to be particularly useful for the discovery of new drugs (Bibi et al. 2017), the green macroalga *Ulva mutabilis* and its bacteria increased our insights in bacteria-induced morphogenesis (Wichard 2015), etc. Yet, biological diversity is enormous and requires the study of many models before generalizations can be made (e.g. O'Brien et al. 2019). A particularly promising avenue for studies relying on model systems is to again expand beyond the one-on-one models to more natural scenarios (Wichard & Beemelmanns 2018), allowing us to assess the ecological relevance of discoveries made.

Diatoms and their bacteria

Diatoms are the most successful group of eukaryotic microalgae in today's oceans: they are responsible for about 40% of marine primary production and fulfil a crucial role in carbon and nutrient cycling and as the base of marine food webs (Armbrust 2009; Bowler et al. 2009). Especially during the last decade, there is increasing evidence that diatoms are involved in complex interactions with heterotrophic bacteria (Amin et al. 2012; Figure 1) and that these interactions might be one of the reasons behind their ecological and evolutionary success (Bowler et al. 2008).

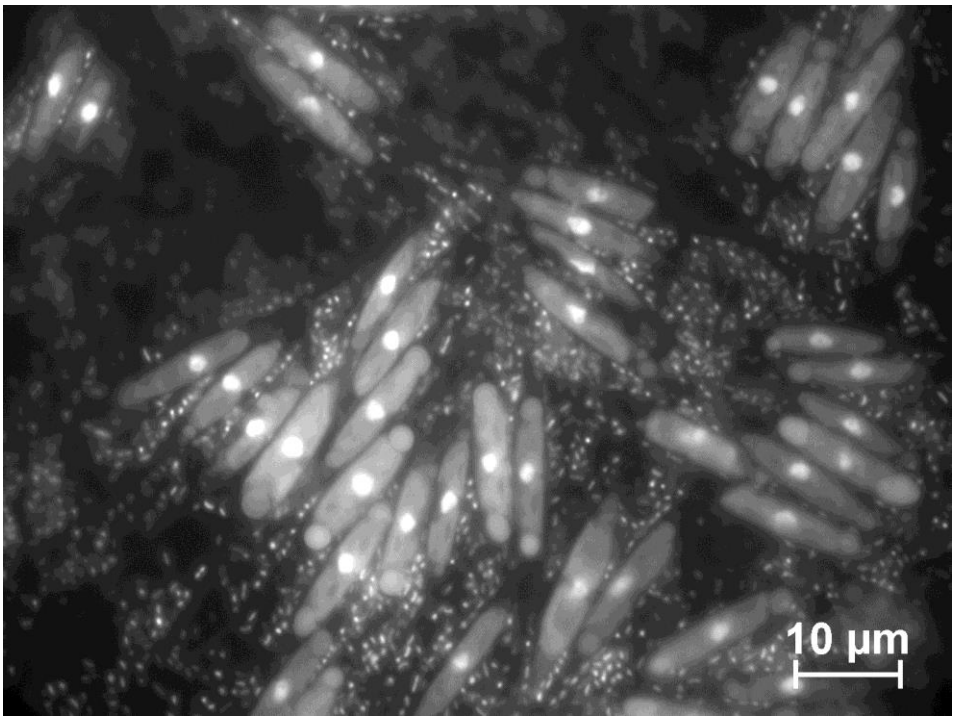

Figure 1: diatoms and their bacteria. A fluorescent image of a DAPI stained xenic *Cylindrotheca closterium* culture. The diatoms are easily distinguishable from the much smaller bacteria by their large nucleus and autofluorescent chloroplasts, Picture taken by F. De Boever.

The influence of the diatoms on the bacteria is largely mediated by exudates (Bohórquez et al. 2017; Bruckner et al. 2011; Doghri et al. 2017; Gärdes et al. 2011). By releasing a portion of their photosynthetically fixed carbon into their bacterial community, diatoms provide bacteria with an organic carbon source (Mühlenbruch et al. 2018;Taylor et al. 2013) and potentially impact the structure of their microbiome (Haynes et al. 2007; Taylor et al. 2013). Differential exudation, commonly observed in diatoms (Underwood & Paterson 2003; Wolfstein & Stal 2002), might be a way to steer interactions with bacteria and fit their microbiome to their most current needs (Sasse et al. 2018; Scheuring & Yu 2012). However, this strategy necessitates diatoms to invest costly resources in order to exert sufficient control on its surrounding bacterial community for it to be beneficial. For plants, which sometimes release up to one fifth of the photosynthates as root exudates to regulate their soil microbiome (Achouak & Haichar 2013), it has been shown that extensive host control is limited to situations where the cost to control the microbiome is less than potential rewards for the host (Revillin et al. 2016). In diatoms, such trade-offs have not yet been investigated.

The co-occurrence of diatoms and bacteria over evolutionary time-scales has left its imprint on both organisms. The genomes of diatoms contain hundreds of bacterial

genes (Armbrust et al. 2004; Bowler et al. 2008), which have provided them with novel metabolic possibilities (Bowler et al. 2008). Concurrently, diatoms depend on bacteria for vitamins (Kojadinovic-Sirinelli et al. 2018; Ellis et al. 2017), iron (Sanchez et al. 2018) and many other metabolites (Cirri & Pohnert 2019). Bacteria have evolved to metabolize some of the most complex algal exudates (Luo & Moran 2014) and actively migrate towards diatoms (Sonnenschein et al. 2012; Smriga et al. 2016). As new technologies, which allow a detailed molecular characterisation of the interactions between diatoms and bacteria (Cooper & Smith 2015), are becoming more widely available, the genomic basis of these interactions will be further unravelled. Such technologies include (meta)transcriptomics (Amin et al. 2015), metabolomics (Stahl & Ullrich 2016), (meta)genomics (Kojadinovic-Sirinelli et al. 2018), *in vivo* expression technology (Torres-Monroy & Ullrich 2018) and combinations thereof. For instance, through transcriptomics and genomics, Amin et al. (2015) showed how a *Sulfitobacter* stimulated diatom cell division by secreting auxin, which it synthesised from diatom-secreted endogenous tryptophan.

Despite ample evidence of diatoms and bacteria being evolutionary intertwined, the evolutionary trajectory of diatoms and their associated bacteria remains unclear. Coevolution has never been properly evaluated, as was for instance done for seaweeds in Hollants and colleagues (2013) by comparing phylogenies of the host and the bacteria. Based on a transplant experiment where bacteria were shown to be mutualistic to their native algal host but parasitic to a foreign diatom, Sison-Mangus et al. (2014) suggested coevolution between partners. However, evidence for coadaptation does not necessarily imply coevolution (Mazel et al. 2018). Other studies have shown that associations between bacteria and their host are highly ephemeral (Kaczmarska et al. 2005; Sapp et al. 2007a) and that diatoms often do not have bacteria attached to them (Crenn et al. 2018). Such findings suggest that stochastic or ecological processes may be driving diatom-bacteria associations rather than coadaptation or coevolution.

Regardless of what the underlying mechanisms are, the associations between diatoms and bacteria are often remarkably specific. Different diatom species generally harbour different bacterial communities (Behringer et al 2018; Grossart et al. 2005; Sapp et al. 2007b) and even closely-related diatoms tend to differ in their microbiomes (Sison-Mangus et al. 2014). The effects of bacteria on the growth of their algal host can be

equally specific (Paul & Pohnert 2013; Sison-Mangus et al. 2014). It is as yet unclear to what degree the specificity is influenced by other factors, such as environmental conditions (Grossart and Simon 2007) and historical processes (e.g. geographic differences in bacterial source pools and priority effects) (Ajani et al. 2018; Lindström & Langenheder 2012; Sörenson et al. 2019). It is clear that our knowledge about diatom-bacteria associations is still in its infancy. Innovative approaches are needed, whereby the eco-evolutionary dynamics of these associations are investigated in the appropriate community ecological framework, to assess how these dynamics can lead to the generally observed specificity.

Diatoms and their bacteria are a convenient model to explore host-bacterial interactions as evidenced by various recent publications (Cirri et al. 2019 and references therein). Countless diatom strains are available in culture, where they can be maintained for long periods of time by means of cryopreservation (Stock et al. 2018). Their growth rate can be relatively easily quantified and compared between treatments (e.g. Behrenfeld & Boss 2006; Appendix I), whereby different species often can be distinguished based on their morphological characteristics. In addition, growth, in the form of asexual (clonal) reproduction, is relatively high for many commonly used species (Edwards et al. 2015), allowing for rapid upscaling and consecutive experiments. Several axenisation protocols have successfully been developed (Bruckner & Kroth 2009; Han et al. 2016; Scholz 2014) and once axenic, diatom cultures can generally be maintained in that state for at least several months (Scholz 2014). Likewise, a large part of the bacteria associated with diatoms can be maintained as pure cultures, allowing full experimental control of these associations (Behringer et al. 2018; Grossart et al. 1999; Kang et al. 2008). Lastly, the complexity of the diatom as a habitat for bacteria is relatively low and associated bacterial diversity is usually also low (Kaczmarska et al. 2005) compared to for instance the human oral microbiome (Welch et al. 2016), making interpretation of results more straightforward. The duality between bacteria and their algal host (Bratbak & Thingstad 1985) makes them particularly useful to study the eco-evolutionary dynamic: bacteria depend on the exudates of the diatoms (Taylor et al. 2013), whilst simultaneously competing with them for nutrients (Diner et al. 2016), forcing coexisting partners to find a balance between competition and mutualism.

Various diatom species have been used to study diatom-bacteria interactions (table 1), including many belonging to common planktonic genera such as *Thalassiosira*,

Leptocylindrus, *Coscinodiscus*, and *Skeletonema* (Ajani et al. 2018; Sapp et al. 2007a; Schäfer et al. 2002). Few diatoms have received as much attention as those belonging to the genus *Pseudo-nitzschia* (Bates et al. 1995 & 2004; Guannel et al. 2011; Sison-Mangus et al. 2014), largely due to the link between bacteria and domoic acid production by these diatoms. Since this neurotoxin might also be involved in the structuring of the diatom-associated bacterial community (Guannel et al. 2011), this genus has proven a useful system to investigate diatom-bacteria interactions. However, the influence of toxin also makes findings from this system more difficult to extrapolate to other diatoms. Benthic diatom-bacteria interactions have thus far not intensively been studied in the lab. The most diverse assembly of benthic diatoms was studied by Bruckner et al. (2008 & 2011). Notably, these diatoms were all isolated from a fresh water system. A *Halamphora* and *Entomoneis* strain were shown to grow better prior to an antibiotics treatment (Jauffrais et al. 2017). Recently, the growth response of *Haslea ostrearia* in relation to bacteria was reported (Lépinay et al. 2018). This is one of the very few examples were a marine tychoplanktonic (benthic and planktonic) diatom has been used in the lab to investigate its relation with bacteria.

Table 1: A decade (2009-2019) of studies on diatoms and their associated heterotrophic bacteria. A non-exhaustive list of studies focussing on (A) the factors influencing the composition of bacterial community associated with diatoms and (B) the growth response of diatoms towards heterotrophic bacteria. (Bacterial Community Composition = BCC).

A

study	diatom genera	findings
Ajani et al. (2018). Frontiers in microbiology, 9, 2758.	*Leptocylindrus*	strong effect of the isolation process on the BCC
Bagatini et al. (2014). PLoS One, 9(1), e85950.	*Aulacoseira* & 2 cyanobacteria	cyanobacteria have a different BCC compared to *Aulacoseira*
Baker & Kemp (2014). Aquatic Microbial Ecology, 72(1), 73-88.	*Thalassiosira* & other marine phytoplankton	substantial variation in the BCC of single algal cells
Baker et al. (2016). Env. Microb. Reports 8(5), 917–927.	*Chaetoceros*	nutrient concentrations and host growth stage effect BCC
Behringer et al. (2018). Frontiers in microbiology, 9, 659.	*Asterionellopsis & Nitzschia*	species specific BCC which is stable over time
Crenn et al. (2018).Frontiers in microbiology, 9:2879.	*Thalassiosira & Chaetoceros*	species specific BCC and strong effect of isolation
Eigemann et al. (2013). FEMS Microb. Ecol., 83: 650–663.	*Stephanodiscus* & a green algae	species specific BCC overrule environmental influences
Guannel et al. (2011). Aquat Microb Ecol 64, 117–133.	*Pseudo-nitzschia*	species specific BCC
Kimbrel et al. (2019). Algal Research, 40, 101489.	*Phaeodactylum* & an eustigmatophyte	selection of the algae results in reproducible community assembly
Kojadinovic-Sirinelli et al. (2018). Env. Microb., 20(10), 3601-3615.	*Asterionella*	taxonomic and functional characterisation of the BC
Majzoub et al. (2019). FEMS microb. Ecol., 95(6), fiz060.	*Thalassiosira*	presence of another bacterium affects BCC
Mikhailov et al. (2018). Acta Biologica Sibirica. 4(4), 89–94.	*Synedra, Asterionella* & *Fragillaria*	description of the BCC
Paul et al. (2012). Mar. Drugs 2012, 10, 775-792	*Skeletonema* with others	strain specific effects on the BCC
Samo et al. (2018). Env. Microb., 20(12), 4385–4400.	*Phaeodactylum* & green alga	BCC characterisation and optimalisation for increased algal yield
Schaum (2019).Limnol. Oceanogr. 64, 441–460.	*Thalassiosira*	BCC is correlated to biofilm formation of the diatom
Sison-Mangus (2013). The ISME journal, 8(1), 63.	*Pseudo-nitzschia*	strain specific BCC linked to algal toxin production

B

study	diatom genera	bacteria	findings
Bigalke & Pohnert (2019). MicrobiologyOpen, e818.	*Chaetoceros & Skeletonema*	*Kordia*	*Kordia* is only algicidal to *Skeletonema*
Bruckner et al. (2011). Env. Microb., 13(4), 1052-1063.	12 different benthic diatoms	4 bacterial isolates	differential impact of bacteria and their spent medium on diatom growth
van Tol et al. (2016). The ISME journal, 11(1), 31.	*Thalassiosira* and other diatoms	Croceibacter	*Croceibacter* inhibits cell division
Amin et al. (2015). Nature, 522(7554), 98.	*Pseudo-nitzschia*	*Sulfitobacter*	Sulfitobacter enhances diatom growth through auxin production
Behringer et al. (2018). Frontiers in microbiology, 9, 659.	*Asterionellopsis & Nitzschia*	10 different bacteria	several growth promoting and inhibiting bacteria
Paul et al. (2012). Metabolomics, 9(2), 349-359.	*Thalassiosira*	*Dinoroseobacter*	impact on algal exudates but not on the algal growth
Cirri et al. (2018). FEMS Microbiology Ecology, 94(11), fiy161.	*Seminavis*	4 bacterial isolates	Impact on growth & sexual reproduction of *Seminavis*
Durham et al. (2017). Env. Microb., 19(9), 3500-3513.	*Thalassiosira*	*Ruegeria*	algal growth enhancement
Han et al. (2016). Biol Res-Thessaloniki 23:8.	*Cylindrotheca* & other algae	*Muricauda*	algal growth enhancement under mixotrophic conditions
Jauffrais et al. (2017). J. of Exp. Mar. Biol. and Ecol. 495: 65–74.	*Halamphora* & *Entomoneis*	natural community	xenic growth higher than antibiotic treated algae
Lépinay et al. (2018).Algal Research 31, 395–405.	*Haslea*	28 bacterial isolates	general growth enhancement
Paul & Pohnert (2013).PLoS ONE 8(3), e57577.	*Chaetoceros & Skeletonema*	*Kordia*	*Kordia* filtrate is only algicidal to *Skeletonema*
Paul & Pohnert (2011).PLoS ONE 6(6), e21032.	*Chaetoceros,*Skeletonema*, Phaeodactylum*	*Kordia*	*Kordia* is not algicidal to *Chaetoceros*
Sison-Mangus (2013). The ISME journal, 8(1), 63.	*Pseudo-nitzschia*	7 bacterial isolates	bacteria beneficial to own host but not to foreign host
Wang et al. (2014). Allelopathy Journal, 33(1).	*Thalassiosira*	*Marinobacter*	algal growth inhibition requires contact

Considering the bias towards planktonic model diatoms to study bacteria-diatom interactions, it should be no surprise that most knowledge on bacteria-diatom interactions to date relates to planktonic systems and very little is known about the relevance of these interactions in benthic environments. In intertidal environments, diatoms build phototrophic biofilms (Van Colen et al. 2014). These biofilms host complex consortia of microorganisms, composed of bacteria, archaea (McKew et al. 2011), viruses (Montanié et al. 2015), and unicellular algae and grazers (Sahan et al. 2007). The biochemical composition of the biofilm matrix is equally complex, representing a conglomerate of various biopolymers such as carbohydrates, proteins, lipids, nucleic acids, humic substances, and combinations hereof (Gerbersdorf & Wieprecht 2014). The biofilms are important for the functioning of coastal habitats as they regulate nutrient fluxes and stabilize the sediments (Van Colen et al. 2014). Diatoms are often the dominant primairy producers of these biofilms (Underwood & Kromkamp, 1999) and heterotrophic bacteria the foremost remineralizers of organic matter (Van Colen et al. 2014). As diatoms and bacteria live in close proximity with each other in these biofilms, interactions can be expected to be intimate and intense as evidenced by the rapid carbon transfer from diatoms to bacteria (Middelburg et al. 2000). Changes in composition and biomass of the benthic microalgal communities have been linked to shifts in bacterial activity and densities (Bolhuis et al. 2013; Decleyre et al. 2015; Lavergne et al. 2017). The opposite is also true as the diatoms community structure altered after changes were induced in the bacterial community (D'Costa & Anil 2014). Despite being clearly connected, the relation between bacteria and diatoms in these biofilms, are not well understood. Part of the connection between both organisms is unquestionably being driven by extracellular polymeric substances (EPS; Bruckner et al. 2011; Haynes et al. 2007). EPS is released in copious amounts by benthic diatoms and function as a major carbon and energy source for the bacteria (Taylor et al. 2013). The composition of diatom secreted EPS will be influenced by the bacteria (Bruckner et al. 2011) and in turn can effect certain specific bacteria (Taylor et al. 2013). If these EPS-based dynamics or other, yet unknown, mechanism lead to species-specific diatom-bacteria associations as generally observed in pelagic settings remains to be seen.

General aims and outline of the thesis

The general objective of this thesis was to obtain a better understanding of the assembly processes that shape the structure of diatom-bacteria consortia in marine intertidal benthic environments, with focus on the importance of bacteria-host coevolution and the influence of the environment and the environmental bacterial pool on the associations between bacteria and the host. We wanted to verify (1) how specific these interactions can be, i.e. if also closely related diatoms differ in their associations and interactions with bacteria and how this is reflected in the diatom-associated bacterial community and the algal growth response, and (2) if this specificity is the result of a shared evolutionary history between diatoms and their bacteria (coevolution) or rather reflects an ecological dynamic. Lastly, (3) we were interested in the relevance of host-specificity on the functioning of both bacteria and diatoms in intertidal benthic environments.

More specifically, we aimed to (Figure 2):

- Identify the bacteria shared between closely related diatom strains from the *Cylindrotheca closterium* species complex and quantify the influence of the host and environment in structuring the *C. closterium* associated bacterial community. To this end, we characterised and compared the bacterial communities associated with a diverse array of *C. closterium* strains, isolated from different localities along the North Sea coast (Chapter II).
- Evaluate the degree of specificity in the interactions between benthic diatoms and their associated bacteria by screening the effect of diatom-derived bacterial isolates on the growth of different *Closterium* strains (Chapter III).
- Evaluate the assembly process of diatom-associated bacterial communities in experiments in which we added natural bacterial inocula to axenic diatoms. By using sympatric and allopatric inocula, we wanted to test if the diatoms would be able to assemble a host-specific, beneficial microbiome, even if the bacterial community did not contain any naturally co-occurring bacteria (Chapter IV).
- Test if the bacteria-diatom associations could impact the structure and productivity of a benthic diatom community. This was done by growing different benthic diatom species, as monoculture and cocultures, with and without bacteria (Chapter V).

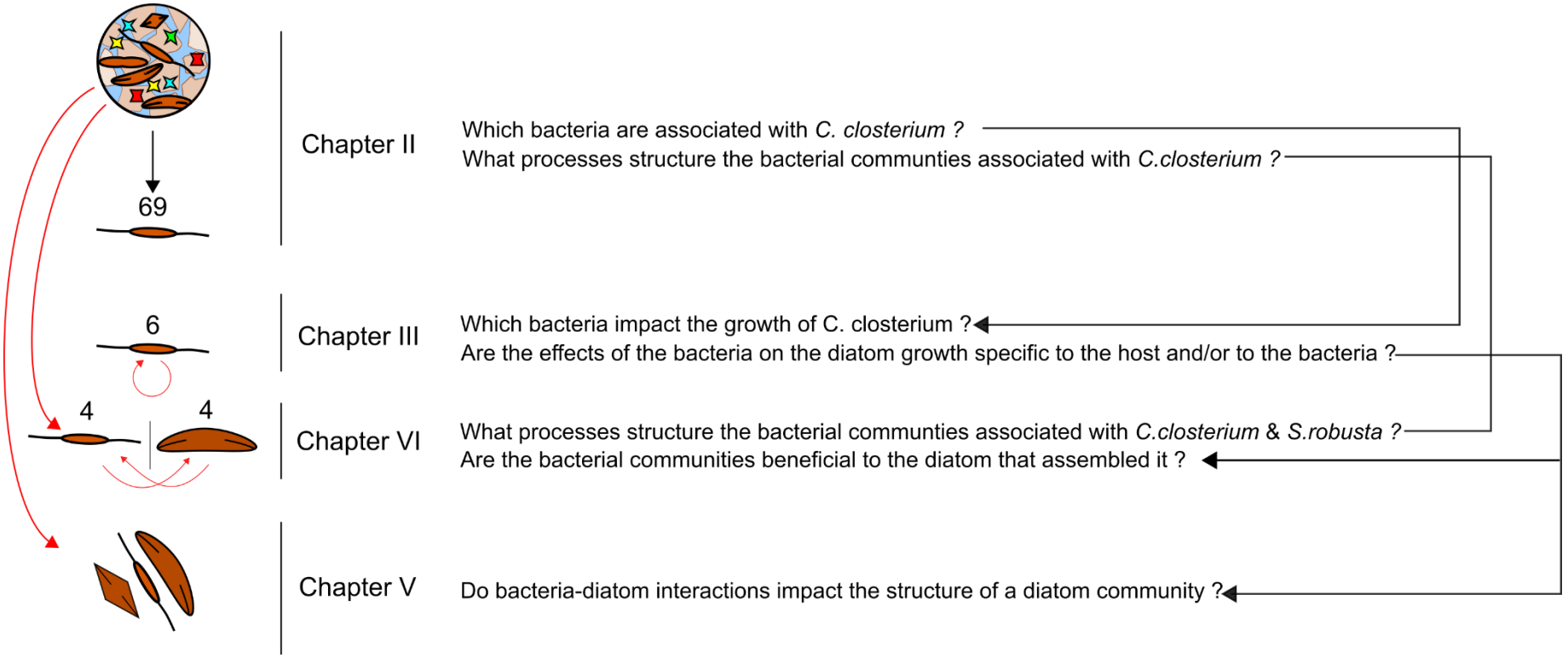

Figure 2: Thesis overview. On the right: a schematic representation of the aims and how these are connected across chapters. On the left, a representation of the diatoms and bacteria used in every chapter (red arrows indicate from where the bacteria orginated & the number of diatom strains used in a study is indicated above the diatom): in chapter II *C. closterium* cells were isolated from environmental samples; in chapter III bacteria, isolated from diatoms, were cocultured with axenic diatoms; in chapter VI bacteria from environmental samples were added to diatoms after which they were exchanged between diatoms & in chapter V bacteria from an environmental sample were added to a mixed diatom community

In this work, we focussed on two benthic model diatoms: *Cylindrotheca closterium* and *Seminavis robusta*.

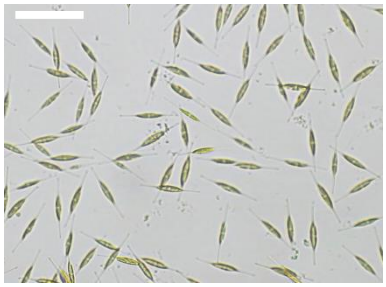

Figure 3: Light microscopic picture of living *C. closterium* WS3_7 culture. Scale bar = 10µm

Cylindrotheca closterium (Ehrenberg) Reimann and Lewin 1964 (Figure 3) is an ecologically important diatom species, which is widely distributed in numerous high and low latitude marine to brackish water regions, as well as a major component of pelagic aggregates and inside sea-ice communities (Underwood & Kromkamp 1999; von Quillfeldt et al. 2003; Najdek et al. 2005). It has been widely used as a diatom model system to study diatom ecophysiology, including the production and function of extracellular polymeric substances (De Brouwer et al. 2005; Pletikapić et al. 2011), movement (Apoya-Horton et al. 2006) and anti-oxidative defence (Rijstenbil 2005). The species was repeatedly shown to represent many (pseudo)cryptic species (Li et al. 2007; Vanormelingen et al. 2013). Many different strains were used in this work, spanning a large part of the known genetic diversity of this species (Figure 4). Appendix II is dedicated to study this genetic diversity and in particular the link between phylogeny and the ecophysiology in this complex.

Figure 4: RbcL based phylogeny of *C. closterium*. This maximum likelihood based phylogeny contains several of the *C. closterium* strains used in this work. The purple strains were a subset of the strains used in chapter II and some were also used in chapter III. WS3_7 (indicated with a *) was also used in chapter IV and chapter V. The black strains have been used in Appendix II. Several of the clades match those previously delineated by Vanormelingen et al. (2013), as indicated on the right of the phylogeny. The maximum likelihood tree was constructed using RAxML (GTR model, partitioned for each codon position) with the C. fusiformis strain IIDO2 set as outgroup. Bootstrap support is indicated.

Seminavis robusta D.B.Danielidis & D.G.Mann 2002 (Figure 5) is an equally important benthic model diatom, used to study motility, life cycle regulation and sexual reproduction (Bondoc et al. 2016; Gillart et al. 2008; Moeys et al. 2016). The genetic diversity in this species is also markedly high (De Decker et al. 2018), but only strains from within one clade (Clade I sensu De Decker et al. (2018)) were used in this study.

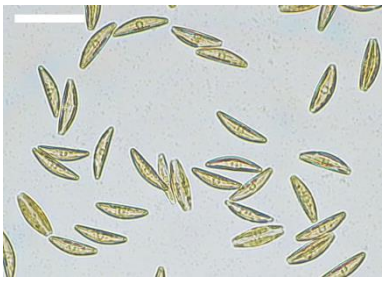

Figure 5: Light microscopic picture of living S. robusta 85A culture. Light. Scale bar = 10µm

Investing the effects of the bacteria on their algal hosts and vice versa, requires an interdisciplinary approach. Two methods in particular have been pivotal to the success of this research: Imaging - Pulse Amplitude Modulated fluorometry and high-throughput amplicon sequencing.

Pulse Amplitude Modulated (PAM) fluorometry measures the *in vivo* chlorophyll a fluorescence (Schreiber 2004) and therefore allows to non-invasively obtain information on the state, productivity and biomass of diatoms (Consalvey et al. 2005). An imaging-PAM fluorometer is equipped with a camera, which makes it possible to distinguish the fluorescence of neighbouring wells of a multiwell plate. As many of the experiments within this dissertation required the simultaneous comparison of many different treatments, imaging-PAM fluorometery was expected to be the most convenient method. Before this work, the suitability of Imaging-PAM fluorometry for diatom growth monitoring had not yet been rigorously tested. As the reliability of growth estimations was essential to our work, the effects of the growth phase transitions (Forster and Martin-Jézéquel, 2005; Bender et al., 2014) were of particular concern to us. In addition, differences between the diatoms and medium used within this study should not influence the reliability of the growth estimates. This was evaluated in appendix I, by assessing the linearity between Imaging-PAM based biomass proxies to cell counts and chlorophyll a concentrations.

High-throughput (HTP) sequencing has revolutionised many fields in biology including microbiome research (Ji & Nielsen 2015). The continuing advances along with rapidly decreasing costs of the DNA sequencing technologies (Goodwin et al. 2016) now allow us to address questions in diatom-bacteria research which we previously could not. For instance, the quantification of a phylosymbiotic signal, whereby the microbiome

structure changes in a way that reflects host phylogeny, requires the comparison of numerous well-characterised diatom-associated communities (Ross et al. 2018) which was until recently beyond the scope of most studies. Despite its many strengths, HTP sequencing also has considerable limitations, such as the large methodological variability between studies which often hampers comparison between them (Clooney et al. 2016). More importantly, the available universal 16S rDNA primers used to characterise bacterial communities are imperfect: they fail to amplify all 16S rDNA species with equal efficiency (Takahashi et al. 2014). Certain bacteria will therefore be overrepresented in an 16S rDNA HTP sequencing dataset whilst other might be missing. It therefore remains essential to analyse such datasets with caution and compare results with for instance culture-based approaches (Tytgat et al. 2014). Nonetheless, HTP 16S rDNA sequencing is one of the key tools for studying microbial diversity (Sambo et al. 2018) and has been essential in obtaining the results presented in this dissertation.

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Chapter II: Getting to the core of the bacterial microbiome in the diatom *Cylindrotheca closterium*

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Getting to the core of the bacterial microbiome in the diatom *Cylindrotheca closterium*

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Abstract

Ecological processes such as dispersal, selection and ecological drift structure host-associated microbiomes. Together, these processes will determine which bacteria will persistently be associated with the host (the core microbiome) and which are more transiently associated with the host (the variable microbiome). We investigated taxonomic, phylogenetic and functional variation within and between bacterial microbiomes of the marine diatom *Cylindrotheca closterium* using a large number of isolates obtained from different locations and environments. Selective recruitment by the host and consistent co-occurrences between bacteria on the one hand, and functional lottery dynamics on the other confirmed the importance of both deterministic and neutral processes in structuring the diatom microbiomes. The core microbiome, taxonomically restricted to *Roseobacter* members and functionally dominated by heterotrophy, was the result of selection imposed by the host but also a likely artefact of the diatom isolation process itself.

Introduction

All organisms live together with bacteria. If there were no selective forces, these microbiomes would reflect what is present in their surroundings. In reality, this is rarely the case as interactions between host, environment and bacteria structure the microbiome on and within the host (Adair and Douglas 2017). The bacterial niches provided by the host differ between host species (Chiarello et al. 2018; Easson and Thacker 2018), but can also depend on the condition and state of the host (Bourne et al. 2008; Ding and Schloss 2014; Pflughoeft and Versalovic 2012). The environment in which the host lives can be equally important (Muletz Wolz et al. 2017). Taken together, influence of host and environment results in the selection of specific bacteria, generally considered to be beneficial for the host.

The specific bacterial enrichment results in a core microbiome: a set of bacteria consistently found with its host. Identifying this core set is an important step in understanding the ecology of the host associated bacterial community as these bacteria are likely critical to the proper functioning of the host-associated microbiome (Shade & Handelsman 2012). Furthermore, deviations in the core microbiome could be indicative for an altered physiological state of the host (Turnbaugh et al. 2009). There has been much debate on how best to define the core microbiome. The frequency at which bacteria have been detected with their host has commonly been used as a criterion (Hernandez-Agreda et al. 2017), but also other criteria have been applied such as the enrichment factor as compared to the environment (Ruiz-Gonzalez et al. 2017) or the position of taxa within an interaction network of host-associated bacteria (Toju et al. 2018). It has been argued that rather than on the taxonomic composition, the functional makeup of the microbiome should be considered (Lemanceau et al. 2017). The core microbiome is complemented by the variable microbiome, which generally comprises the majority of the taxa and is often ignored (Adair and Douglas 2017). This variability can be present between hosts, but also within a host, both spatially and temporally (Caporaso et al. 2011). The variable microbiome can consist of commensals and short-term visitors (O'Brien et al. 2019) which are largely trivial to the host, but the variable microbiome can also prove useful to the host by providing extended metabolic functions in contrasting environments (Candela et al 2012; Goh et al. 2013), thereby potentially alleviating the effects of environmental stressors on the host.

Our aim was to quantify the relative importance of the host, the bacteria present and their environment in shaping the bacterial microbiome of a diatom and to relate these factors to the existence of a core microbiome. Diatoms are one of the most abundant and diverse groups of eukaryotic microalgae (Armburst 2009) and their interactions with bacteria are likely to be one of the reasons behind their ecological and evolutionary success (Bowler et al. 2008). Different diatom species are assumed to have unique bacterial communities (Behringer et al. 2018; Crenn et al. 2018; Grossart et al. 2005; Guannel et al. 2011), although this has been disputed based on the high variability observed within microbiomes of the same diatom species (Ajani et al. 2018; Kaczmarska et al. 2005; Sapp et al. 2007). This variability has been attributed to the location and season the diatoms were collected (Ajani et al. 2018), the conditions in which the diatoms were cultivated (Baker et al. 2016; Crenn et al. 2018; Kaczmarska et al. 2005; Sapp et al. 2007), and the interactions between the bacteria in the microbiome themselves (Majzoub et al. 2019).

Despite the surging interest in diatom-bacteria interactions (Cirri & Pohnert 2019) and the relative simplicity of these microbiomes, particularly in the unialgal cultures as used in most studies (Crenn et al. 2018), an ecological framework for the diatom microbiome remains to be developed. Such a framework, whereby the impact of host, environment and bacteria present is condensed to fundamental ecological processes such as selection and ecological drift (Vellend 2010), would allow us to disentangle the different facets which shape these microbiomes.

In this study, we focussed on bacterial microbiomes of the marine diatom *Cylindrotheca closterium*, a model diatom (Vanormelingen et al. 2013) that has a high genetic diversity, representing several cryptic species (Li et al. 2007; Stock et al. 2019). It often occurs in high densities both in the water column and on intertidal sediments (de Brouwer et al. 2005; Najdek et al. 2005).

We isolated single diatom cells belonging to this species complex from different locations in order to compare their microbiomes. The isolates were then reared under common garden environmental conditions for 2 growth cycles before characterising their bacterial communities. We anticipated the selection on the bacterial communities by the host and as a consequence of the isolation procedure, to result in microbiomes markedly different from the bacterial source communities (Figure 1). Based on previous reports on the isolation of diatoms (Ajani et al. 2018; Sapp et al. 2007), we

expected the isolation procedure to result in a consistent enrichment of specialised bacteria and thus a core microbiome shared between all *C. closterium* strains. Simultaneously, we expected variability between the microbiomes due to differences between the hosts and as a result of competition between functionally equivalent bacteria. If host selective forces are strong, we would expect to see consistent differences between the microbiota of the different hosts, independent of environmental background, whereby a phylosymbiotic signal could be an important indication for evolutionary conservatism of interactions.

To verify our hypotheses, we characterised the bacterial communities of the hosts and the sampling locations through 16S rDNA high-throughput sequencing. The microbiome core was delineated at the different taxonomic levels in addition to a functionally defined core. The influence of the host, the sampling location and isolation procedure on the variable microbiome was quantified through variation partitioning. By comparing the microbiome structure to those simulated under a null model, we quantified how differences between hosts impact the diversity of the microbiomes.

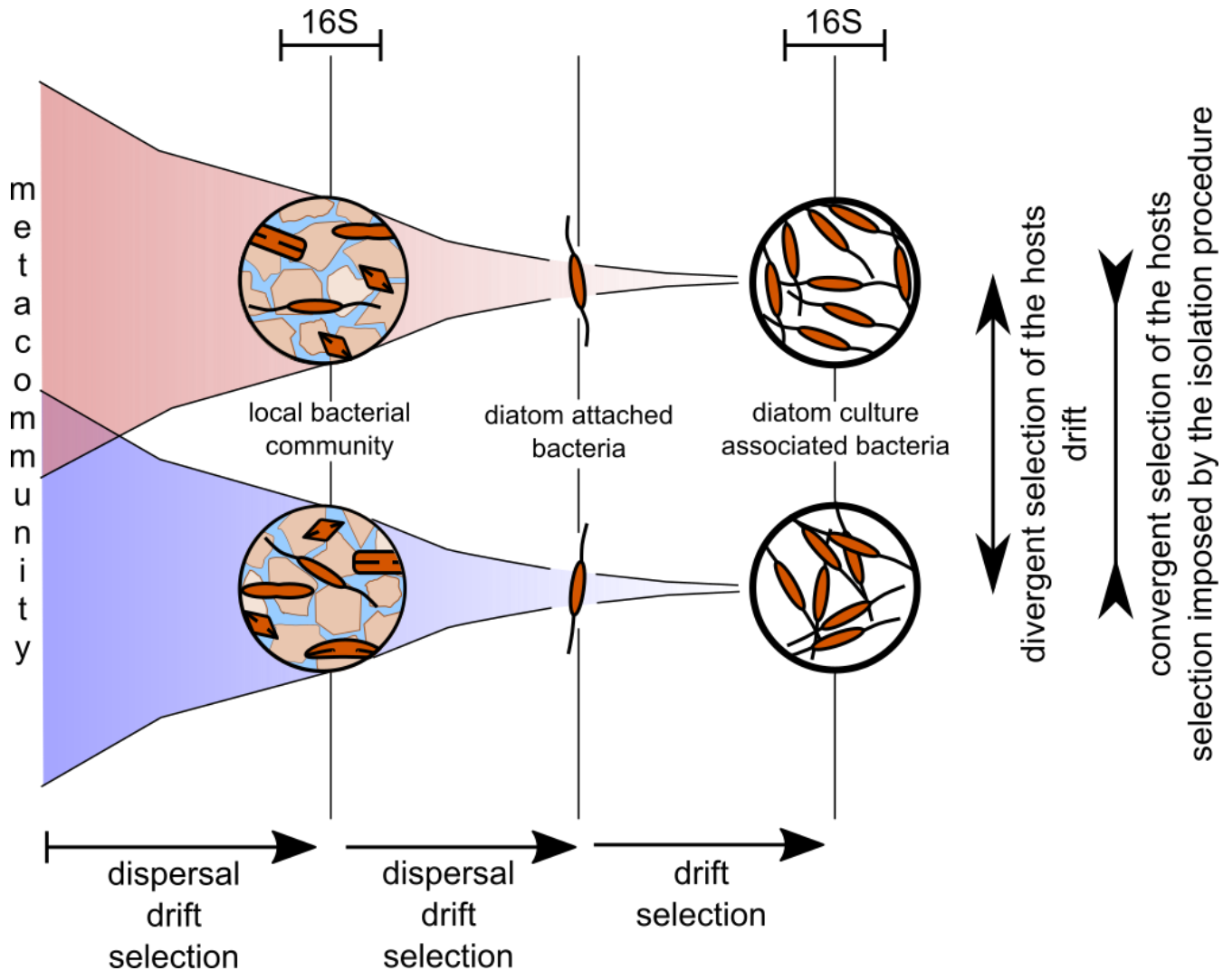

Figure 1: Do the ecological processes lead to a shared bacterial community between *C. closterium* strains? The bacterial communities in the *C. closterium* cultures and from the source samples the diatoms were originally isolated from, were characterised through 16S rDNA high-throughput sequencing. The similarity between bacterial communities associated with the cultured diatoms will depend on the selection imposed by the isolation procedure and the host as well as by the degree of drift and the interactions between the bacteria themselves during microbiome assembly.

Material and methods

Sampling

Water and sediment samples were taken between May and July 2014 from 6 different estuarine locations in the North Sea and English Channel (Table 1).

Table 1: Overview of the diatoms used for this study. The number of isolates is shown for each station. Additional information about the stations is provided including geographical information and the sampled material.

Location	Station	Latitude	Longitude	Sample type	*Cylindrotheca closterium*	Other species
North Holland	NH2	52.93520	5.01815	Sediment	1	
	NH3	52.89533	4.91042	Sediment	6	
Eastern Scheldt	OS4	51.53022	3.94448	Sediment	11	
	OS5	51.52024	3.93640	Sediment	1	
	OSPL1	51.54938	3.86865	Water	1	2 (*Nitzschia* spp.)
	OSPL4	51.53022	3.94448	Water	6	
Bay of the Somme	SO1	50.21445	1.56773	Sediment	2	
	SO3	50.21512	1.56710	Sediment	2	1 (*Navicula phyllepta*)
Wester Scheldt	WS3	51.35053	3.72623	Sediment	4	
	WSPAUL	51.35260	3.73078	Water	2	
	WSTER	51.33710	3.86612	Water	4	
The Zwin	ZW2	51.36550	3.36428	Sediment	18	1 (*Navicula phyllepta*)
	ZWPL4	51.36658	3.36553	Water	2	1 (*Navicula* sp.)
Bay of the Canche	CA1	50.53735	1.59335	Sediment	9	
				total:	69	5

Samples were taken on dry days, with ambient air temperatures above 15 °C. Water samples of the upcoming tide were collected and stored on ice. Sediment samples were taken with 6.4 cm diameter Plexiglas core. The depth of the oxic layer was measured on the core immediately after sampling. The centre (3 cm diameter) of the top centimetre of the core was subsampled and stored on ice. The remaining sediment from the top centimetre, designated for nutrient analysis, grain size analysis and total

organic matter content (%TOM) determination, was immediately frozen until processing. Salinity of the interstitial water and the upcoming water were measured *in-situ* with a hand-held refractometer (Atago).

Sample processing

The sediment that was stored on ice was mixed thoroughly upon arrival in the lab. Half of the sediment was stored at -80 °C for bacterial community analysis. The other half was used to isolate diatoms using the lens tissue method (Moss 1966). For this purpose, the sediment was spread out in a Petri dish (60x15 mm Cellstar® Greiner Bio-one) and covered with two layers of lens tissue (Whatman) and a coverslip (20x40 mm, VWR). After an incubation period of 24 h, at 18 °C with a 12/12 light cycle (20-25 µmol photons/m^2/s), the coverslip was removed and gently rinsed with autoclaved North Sea water (NSW). The attached epipelic diatoms were collected in a Petri dish filled with NSW and allowed to settle for several hours.

Upon arrival in the lab, the water sample was homogenized and subsamples, which were taken for bacterial community analysis and nutrient analysis, were stored at -80 °C and -20 °C, respectively. The rest of the sample was used to isolate diatoms. The diatoms were allowed to settle overnight at 4 °C, in the dark. The next day, some of the precipitate was collected and diluted in a Petri dish filled with NSW. The diatoms were allowed to settle for several hours.

Grain size analysis (median grain size) was done with the Coulter Counter LS Particle Size Analyser. The concentration of ammonium, silicate, nitrate, nitrite and phosphate for (interstitial) water was measured with an automatic chain (SAN plus Segmented Flow Analyzer, SKALAR) after filtration of the samples on Whatman GF/F filters. %TOM was measured by calculating the weight loss after combustion (550 °C for 2 h) of the hot air dried sediment.

Diatom culturing

Single diatom cells were isolated from the prepared Petri dishes by micropipetting (Andersen 2005). Briefly, single cells were resuspended with a needle and brought to the surface from where they were pipetted into a well (96 well plate Cellstar® Greiner) filled with NSW supplemented with F/2 (Sigma-Aldrich). The well plates were placed at 18 °C, with a 12/12 light cycle (20-25 µmol photons/m^{-2}/s^{-1} of cool fluorescent white light) and inspected regularly. Cultures in which contaminants (e.g. flagellates or other

diatoms) were observed were discarded. Once the monoclonal cultures were dense, i.e. the cells covered most of the well, the medium was refreshed prior to the transfer of the culture to a new well (12 well plate Cellstar® Greiner). After 3-4 days, the medium of late exponential cultures were refreshed again before they were harvested. One millilitre of resuspended culture was used for DNA extraction and another millilitre was fixed (final concentration of 4% formaldehyde) for flow cytometer analyses.

DNA analysis

DNA of the diatom cultures and environmental samples was extracted using phenol-chloroform method as in Muyzer *et al.* (1993). For the bacterial community analyses, the V1-V3 hypervariable regions of the 16S rDNA were amplified using pA (AGAGTTTGATCCTGGCTCAG, positions 8-27) and BKL1 (GTATTACCGCGGCTGCTGGCA, positions 536-516) primers as in Tytgat et al. (2016). PCR and library prep were done according to D'hondt et al. 2018. Sequencing was done on an Illumina MiSeq system (300bp paired-end). Artificial mock communities, blanks and duplicate samples were included for quality control. The obtained forward and reverse reads of 16S rDNA sequences were merged using the software program Paired-End Read Merger (PEAR version 0.9.4; Zhang et al 2014). The merged reads were further processed in Usearch8 (Edgar 2013). Clustering was done on the dereplicated reads with the singletons removed. Chimeras were removed de novo and by mapping to the GOLD database. An Operational Taxonomic Units (OTU) table was then constructed using a 3% cut-off. OTUs were classified in MOTHER (Version 1.32.1; Schloss et al. 2009) using the May 2013 GreenGenes training set (DeSantis et al. 2006; Schloss 2010; McDonald et al. 2012). A phylogenetic tree of the OTUs was constructed through agglomerative clustering of sequences in Usearch8. This tree was further used to estimate the phylogenetic distances between OTUs (see below). Functional annotation of the OTUs was done using FAPROTAX (Louca et al. 2016). The taxonomic assignments of the OTUs were hereby mapped to metabolic and other ecologically relevant functions (e.g. denitrification or fermentation), based on the literature on cultured representatives.

The phylogenetic relatedness between the collected algal strains was inferred from two genetic markers. The nuclear ITS region (ITS1-5.8S-ITS2) was obtained for every strain according to Vanelslander et al. (2009). The chlorophyll (chl) 16S sequences were acquired for the 16S MiSeq data: the most occurring sequence in the dereplicated

read files was extracted and manually checked for its resemblance to known chlorophyll sequences. Each marker (ITS and chl 16S) were aligned by ClustalW using MEGA 7. The alignments were manually curated and afterwards joined using SequenceMatrix (version 1.8). Pairwise-distances between the strains were calculated on the concatenated alignment in MEGA7 as p-distances with gaps treated as pairwise deletions.

Cell culture characteristics

Algal cell densities and dimensions were obtained for each culture using an Amnis ImageStream X® Mark II (Millipore) flow cytometer. The LED (brightfield) was set to an intensity of 33.32 mW and the lasers of 642 nm (to detect autofluorescence of the cells) and 785 nm were set to 10 mW and 0.5 mW, respectively. Objects in the samples were acquired until at least a thousand diatom-sided objects were measured. Flow cytometer data was analysed in the Amnis IDEAS 6.2.187.0 software (Millipore). Diatom cells were gated relying on the autofluorescence signal and brightfield aspect ratio. Gates were manually checked and adjusted for every sample using the images required for every object. Apart from diatom cell density, average diatom cell length was calculated using the skeleton mask (to correct for the curvature of the cells) as well as the average diatom cell perimeter.

Data processing

Sequences matching chloroplasts and mitochondria were removed before further analysis. Based on the control samples, read counts below four were set to zero. Samples from diatom cultures with less than 500 reads were discarded and the remaining culture samples (table1) were rarefied to 538 reads. Samples directly from the environment (sediment or water) were rarefied to 2484 reads. These datasets were, as such, used for all further analyses.

The square root transformed communities were used in a Constrained Correspondence Analyses (CCAs). CCAs were performed for the spatial, phylogenetic, morphological and environmental variables separately. The nutrient concentrations were log-transformed prior to the analyses. The spatial variables were constructed using the location from which the samples were taken. Geographical distances between sample locations were calculated and decomposed into PCNM variables (vegan 2.4-4; Borcard and Legendre 2002) which could then directly be used in the CCA. A similar approach was used to construct phylogenetically representative

variables, as the genetic p-distances between *C. closterium* strains were decomposed into PCNM variables. The morphological variables were diatom densities, cell length and perimeter, as obtained from the flow cytometer analyses.

Within each set of variables, the most relevant variables were selected using the stepwise forward selection procedure within CCAs, with the p-value thresholds to include and exclude each variable set to 0.05 and 0.1, respectively. Variables with a variance inflation factor (VIF) above 10 were removed. Variance partitioning was done with retained variables for each set (spatial, morphology, environmental and diatom phylogeny; Supplementary table 1) as explanatory variables in a redundancy analysis ordination (RDA, vegan package). Permutation tests were used to verify significance of the total variance explained by each set of variables in addition to the unique variance explained by each set. The variation partitioning was repeated with the retained environmental and spatial variables grouped as 'source' variables and the phylogenetic and morphological variables grouped as 'host' variables. This variation partitioning was performed with both the relative abundance and the presence/absence transformed bacterial community data. To quantify the joined effect of the isolation procedure and the hosts on the bacterial communities, a CCA was performed on the source and culture samples (both rarefied to 538) together. The analysis was constrained by sample type (source or culture) whilst conditioning for the different locations.

To identify bacterial hub taxa, i.e. bacteria strongly interconnected with the presence of other bacteria (Agler et al. 2016), a partial correlation based network was constructed. Both the relative abundances of the OTUs and the 'source' and 'host' variables retained in the CCAs were used in these analyses. To reduce the number of nodes, and increase power for the network statistics, only the OTUs present in at least five samples were used. Prior to the network analyses, both square root transformed relative abundances and the variables retained in the CCAs were normalised through nonparanormal transformation (huge package, version 1.2.7). A regularized partial correlation network was constructed (estimateNetwork function, in the bootnet package, version 1.1) as illustrated by Epskamp et al. (2018). Briefly, the correlations between nodes were quantified, whilst conditioning on the other nodes, similar to linear mixed effect models, where the relation with fixed effects is considered whilst correcting for random effects. To increase sparsity, and thus interpretability of the

network, the obtained number of correlations (edges in the network) was reduced through regularisation (LASSO; Tibshirani, 1996) with the tuning parameter set to 0.05. Networks stability was assessed through bootstrapping of the data. The strength and centrality of the nodes (variables) in the network was compared to identify the most important nodes.

A conservative randomisation procedure (permatswap with the quasiswap method from vegan 2.4-4) was applied on the 16S rDNA data from the cultures, preserving both the matrix fill (the number of zero occurrences) and the column and row abundance totals. This procedure was constrained within the cultures originating from the same sample in order to maintain structural differences between samples. The randomisation resulted in a thousand simulated 16S datasets, which were structurally compared to the original 16S dataset by means of different α- and β-diversity indices. The Shannon diversity index (*H*) was calculated for every community in all datasets relying on the OTUs and on the corresponding FAPROTAX-based functions. Since the randomisation algorithm maintained the OTU richness, differences of *H* based on the OTUs reflected differences in evenness. For the functional annotations, differences of *H* reflected both differences in richness and evenness. To this extent the richness of the functional annotations was also calculated as the number of functions present in each community. The phylogenetic diversity within every community was calculated as mean pairwise phylogenetic distance (mpd function from the picante package) separating OTUs in a community, not taking the relative abundances of the OTUs in consideration.

The differences between communities within the same dataset were compared through several complementary indices. The Bray–Curtis Dissimilarity index (vegdist function from the vegan package) between all community pairs based on the OTU incidences was calculated in addition to the checkerboard score (C score; C.score function from the bipartite package). The C score (Stone and Roberts, 1990) expresses the strength of co-occurrence patterns between species over the different microbiomes. Variation in bacterial dominance among communities was quantified as the variance in abundance rank (rank assigned to OTUs based on their abundance in a community) for each OTU. The Bray–Curtis Dissimilarity index between communities was also calculated for the presence/absence of the functional annotations. The OTU-based and functional Bray–Curtis Dissimilarity indices were also calculated using the

abundance data. The phylogenetic differences between communities on the other hand were quantified as the mean pairwise phylogenetic distance separating the OTUs in two communities (comdist function from the picante package). This was done both with and without weighting by OTU abundances.

Results

The bacterial communities of diatom cultures and the environmental samples from which they were isolated were characterised through high throughput 16S rDNA amplicon sequencing. The communities in the cultures were markedly different from the environmental samples (Supplementary Figure 1). The rarefaction curves (Supplementary Figure 2) suggest that sufficient sequencing depth was achieved for each sample. The diversity of the bacterial communities from the diatom cultures was thirty times lower than that of the environmental samples (expected species after rarefaction of 8.17±0.49 compared to 246.00±28.57). The diversity in the diatom cultures could not be related to the diatom cell size ($p>0.05$).

The bacterial communities in the *C. closterium* cultures where dominated by Rhodobacterales (Alphaproteobacteria), Campylobacterales (Epsilonproteobacteria) and Flavobacteriales (Figure 2). In fact, all communities contained Proteobacteria (Supplementary Figure 3A), of which *Sulfitobacter* (in 77% of the *C. closterium* cultures), *Loktanella* (in 62% of the *C. closterium* cultures) and *Arcobacter* (in 48% of the *C. closterium* cultures) were most frequently observed. Flavobacteriales were observed in 74% of the *C. closterium* cultures. Bootstrapping of the number of communities suggests that the genera listed here as the most prevalent ones, would have been identified as such when considering approximately ten *C. closterium* microbiomes and upwards (Supplementary Figure 3B). These common genera were also observed with the other diatom species (Table 1): in those diatom cultures where *Sulfitobacter* was detected, *Loktanella* was present in two out of the five cases and *Arcobacter* in one case. The source samples exhibited a higher bacterial variety than algal culture samples, but were also dominated by the same orders (Supplementary Figure 4).

The bacteria in the *C. closterium* cultures were also functionally very different from those in the environmental samples (Chi-Square Test: $p<0.0001$). Of the 42 functions in the environmental samples, 16 were retrieved in the culture samples

(Supplementary Figure 5), chemoheterotrophy, nitrogen respiration, fermentation and the oxidation of sulphur compounds being the most notable ones. Chemoheterotrophy was consigned to all but one of the bacterial communities. Functions present in the sediment samples but not in the cultures included: degradation of complex carbon compounds such as lignins and aromatic hydrocarbons, parasitism and the respiration of sulfur compounds.

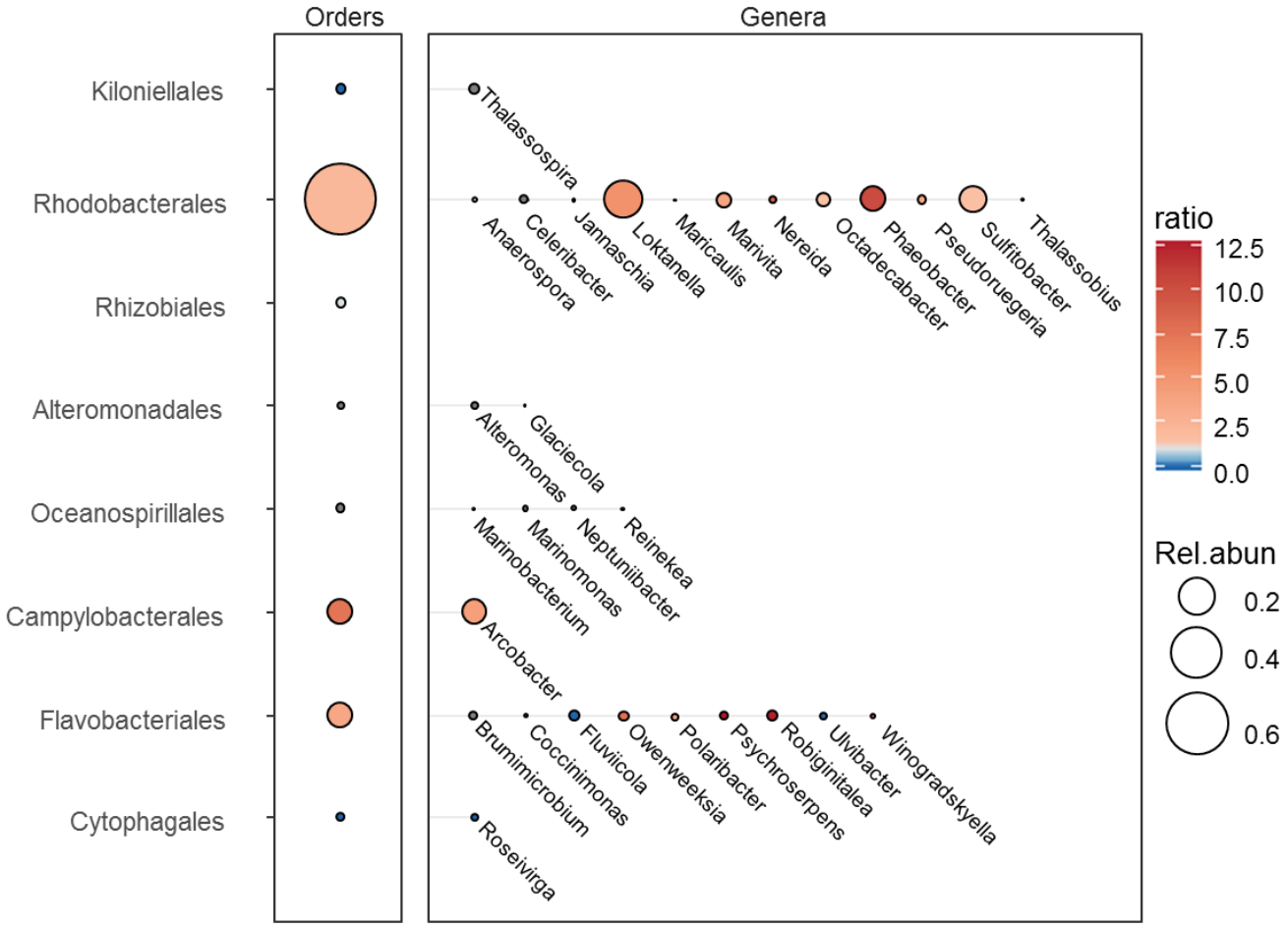

Figure 2: Relative abundance of the bacteria recruited by the *C. closterium* cultures. The size of the sphere is proportional to the average relative abundance across all *C. closterium* strains and the colour represents the ratio between the relative abundance in the cultures to the relative abundance in the matching environmental samples: a red colour being indicative of an enrichment of that bacterial group in the diatom cultures compared to the environment. Only the orders observed in at least 10% of the cultures samples are shown and for these orders, the genera are shown which occur in at least two different diatom samples.

The importance of both the source (environmental conditions and geography) and host (phylogenetic position and cell size) on the structure of the bacterial communities in the algal cultures was compared through variation partitioning (Figure 3; Supplementary Table 1). The host had a less pronounced influence in structuring the bacterial community than the source (Supplementary Figure 1). Only when considering

the relative abundances of the bacteria was the unique contribution of the host significant (9.8% of total variance explained; adj. $R^2_{rel.\ ab}$=3.3%; p=0.019). In that case, the phylogeny of the host (retained phylogenetic PCNMs 1,4,7,12 and 22 out of the 23; adj. $R^2_{rel.\ ab}$=1.9%) and the size of the host (adj. $R^2_{rel.\ ab}$=1%), defined as the mean perimeter of the diatoms, also each had significantly (p= 0.027 & 0.047 respectively) unique contributions to the total variance explained. Neither were significant (p>0.05) when considering only the presence/absence of the bacteria.

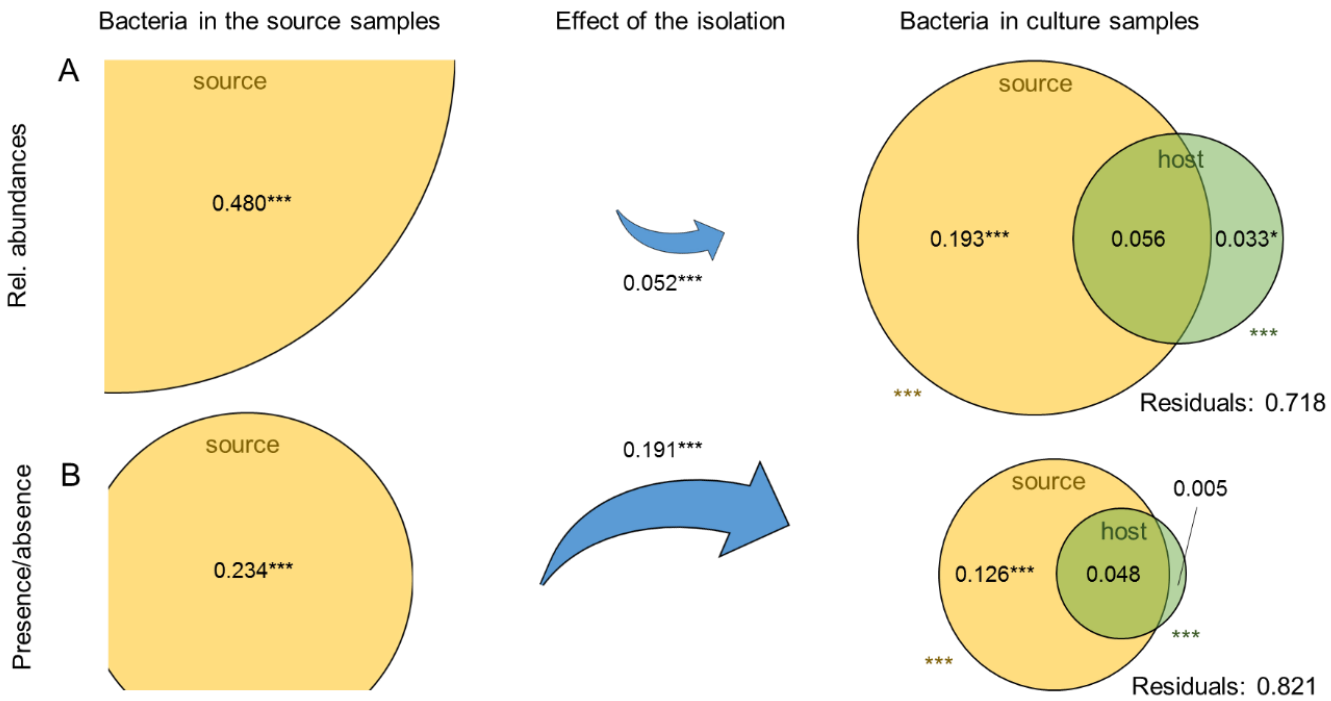

Figure 3: The source has a stronger influence than the host on the bacterial communities in the algal cultures. The sizes of the circles are proportional to the variance in the bacterial communities explained by the respective variables of each group. The Adjusted R^2 values are shown within their respective areas. (A) shows the proportional importance of host and source on the relative abundances of the bacteria whilst (B) shows it for the presence-absence of the bacteria. The significance of the different partitions is indicated with asterisk (*** indicates a p-value of 0.001 or smaller and * indicates a p-value between 0.05 and 0.01). The asterisks outside of the circles specify the significance of the total variance explained by the group; whilst the asterisk next to the adj. R^2 specify significance of the variance uniquely explained by that group.

The variation in the microbiomes explained uniquely by the source, on the other hand, was significant when considering the relative abundances of the bacteria (26.7% of total variance explained; adj. $R^2_{rel.\ ab}$=19.3%; p=0.001) as well as their incidence (22% of total variance explained; adj. $R^2_{rel.\ ab}$=12.6%; p=0.001). In both cases, the unique contributions of geography of the source (retained spatial PCNMs 1 to 4, out of the 7;

adj. $R'^2_{rel.\ ab}$=8.2%; adj. R'^2_{incid}=3.3%) and the environmental conditions of the source (retained variables: planktonic/benthic habitus, salinity and the nitrite and silicate concentrations; adj. $R'^2_{rel.\ ab}$=10.8%; adj. R'^2_{incid}=6.0%) were separately also significant (all p-values ≤0.001).

In an overarching analysis, on both source and culture samples, 3.8% (p=0.001) of the variation in the bacterial communities could be attributed to the sample type (source or culture). The importance of the sample type was much larger when only the presence/absence of bacteria was considered (30.9%; p=0.001).

The presence of the bacteria in the *C. closterium* cultures could be directly related to the abundance of those bacteria in the sample from which the *C. closterium* strains were isolated (the matching source samples): for the three stations from which most of the diatom cells were obtained (CA1, OS4 and ZW2), a significantly (p<0.0001) positive linear relation was found between the relative abundance of the OTUs in the source and the frequency at which they were observed in the algal cultures.

The partial correlation network constructed with the different OTUs and the variables retained in the variation partitioning analyses, proved to be sparse (Supplementary Figure 6) and with mostly the variables and not the OTUs taking up the more central positions. Although a low stability of this network limits the extensive interpretation of this network, it shows that there are no (obvious) hub taxa or consistent co-occurrence patterns between bacteria.

The observed structure in the bacterial communities from the *C. closterium* cultures showed pronounced differences compared to those generated through randomisation (Figure 4, row 1). The OTU-based Shannon diversity was smaller than in all simulations (p≤0.001), indicative for a lower evenness. Since the randomisation algorithm conserves total abundances of each OTU, consistently dominant bacteria would still be dominant in the randomised matrices and communities would thus have a similar evenness. The lower evenness of the original dataset has to be the result of various alternative states in which the dominant OTUs differ between communities. This is supported by a higher variance in abundance rank of the OTUs in the original dataset compared to the simulated datasets (p≤0.001). The mean Shannon index based on functional diversity was much higher in the original dataset than in the simulated datasets (p≤0.001). This coincided with a higher than expected number of observed

functions in the communities (4.88 compared to 4.33±0.02 after randomisation; p=0.001). The phylogenetic diversity within communities (mean pairwise phylogenetic distance), on the other hand, was smaller than expected (p=0.006). The phylogenetic diversity does not take relative abundances into consideration and as such is independent of the lower OTU-based Shannon diversity.

When comparing the differences in presence/absence between communities, similar patterns were observed when considering OTUs, functional composition or phylogenetic diversity (Figure 4, row 2). In all cases, the differences between communities were smaller than expected under the simulations (p≤0.001). The lower OTU-based Bray–Curtis Dissimilarity was reflected by an equally lower than expected C score (p≤0.001), indicative for a more aggregated co-occurrence of species. The latter was also reflected by the predominance of positive correlations between OTUs in the network (Supplementary Figure 6). The lower function based dissimilarity and mean pairwise phylogenetic distance also confirm a positive co-occurrence of the bacteria. When comparing abundances of bacteria between communities, very different trends were observed (Figure 4, row 3). A higher OTU-based dissimilarity and mean phylogenetic distance (abundance weighted mean pairwise phylogenetic distance) were observed compared to the simulations (p≤0.001). The functional dissimilarity however did not differ between the observed and the simulated data.

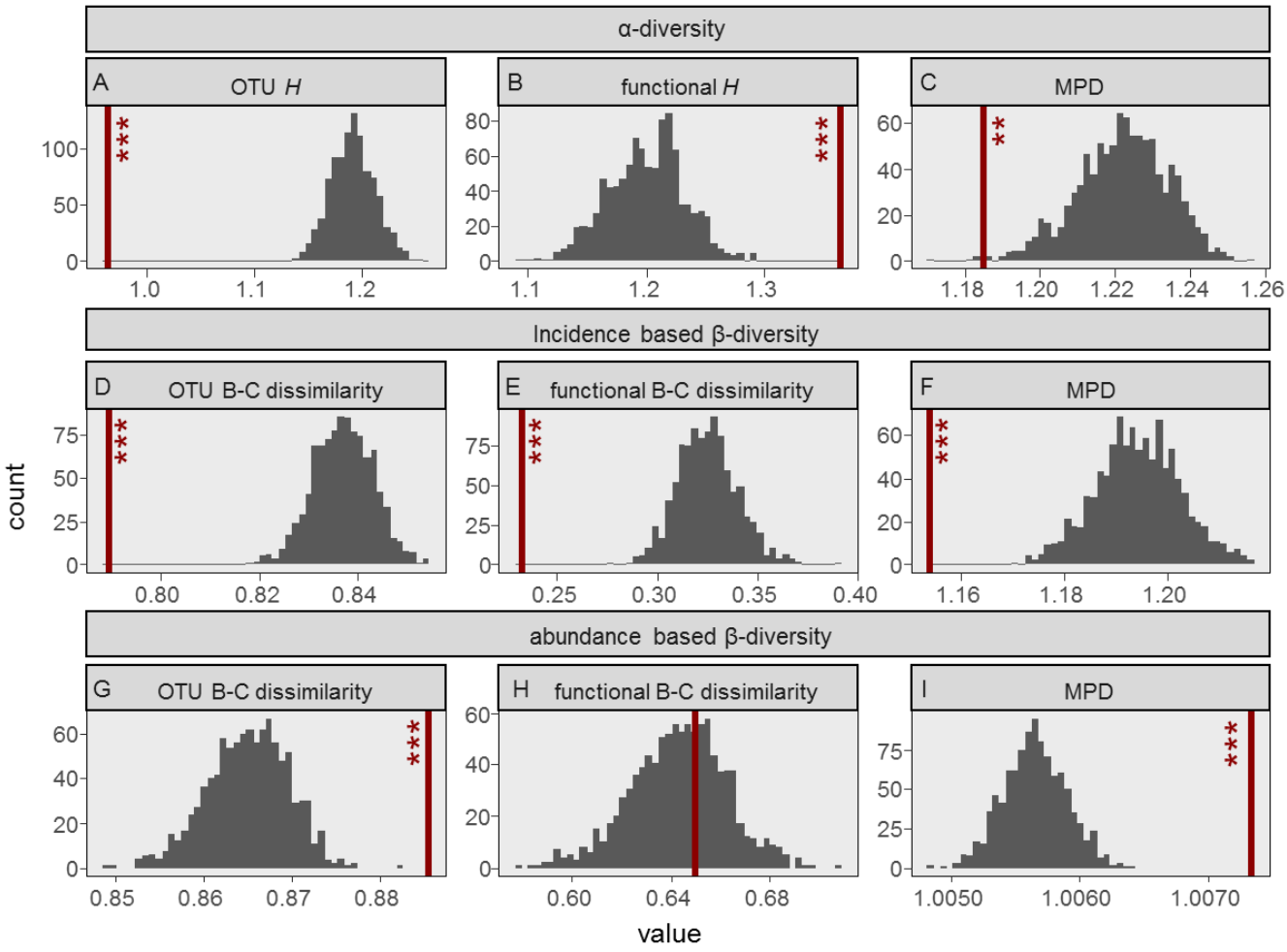

Figure 4: diversity indices of the data compared to those simulated under the null model. The histograms show the distribution of the mean indices for the 1000 randomised datasets (null models) and the red line shows the outcome of the original dataset. The number of occasions that the simulated values were more extreme than those obtained from the original data are indicated by red asterisks (*** none extremer; ** between the one and ten occurrences). The alpha-diversity (within communities) indices are shown in the first row and the beta-diversity (between communities) indices in the two lower rows. From left-to-right then top-to-bottom: (A) the Shannon diversity based on the OTU abundance data, the (B) Shannon diversity based on the abundance of the annotated functions, the (C) mean pairwise phylogenetic distance (MPD) within communities, the (D) Bray–Curtis Dissimilarity based on the OTU incidence data, the (E) Bray–Curtis Dissimilarity based on the presence/absence of the annotated functions, the (F) mean pairwise phylogenetic distance between communities, the (G) Bray–Curtis Dissimilarity based on the OTU abundance data, the (H) Bray–Curtis Dissimilarity based on the abundance of the annotated functions and the (I) mean pairwise phylogenetic distance between communities, weighted by the relative abundances of the OTUs.

Discussion

The core microbiome

The bacteria communities associated with *C. closterium* were comparable to what was previously found in the microbiomes of other diatoms (Amin et al. 2012; Behringer et al., 2018; Ajani et al. 2018) with Proteobacteria and to a lesser extent Flavobacteriia dominating the microbiomes. The most frequently observed genera, namely *Loktanella*, *Phaeobacter*, *Sulfitobacter* and *Arcobacter,* all belonged to the Proteobacteria. Not much is known about the ecology of marine *Arcobacter* (Salas-Massó et al. 2016), but it has been observed with algae before (Kim et al. 2010). The other three genera all are members of the *Roseobacter* clade, a group of Alphaproteobacteria universally found in algal microbiomes (Luo and Moran 2014). These bacteria are particularly well-adapted to interact with their algal hosts, as many members from this clade have been shown to be chemotactic, easily adhere the algae and have the ability to breakdown the complex carbon compounds released by algae (Geng and Belas 2010). They often are beneficial to their host as they can secrete several algal growth-stimulating metabolites (Amin et al. 2015; Luo and Moran 2014).

Previously, other *Roseobacter* clade members were identified as core taxa. A *Roseovarius* sp. was found in all cultures of the cosmopolitan diatom *Leptocylindrus* (Ajani et al. 2018), whilst two other, still unclassified, members were found in all diatoms isolated from the Arabian Gulf (Behringer et al., 2018). This illustrates that members from *Roseobacter* clade are persistently associated with diatoms, but that different clade members can take up this role.

With heterotrophy and several anaerobic processes as central functions, a functional core microbiome could also be defined for *C. closterium*. As the diatom-associated bacteria are largely dependent on the carbon fixed and released by their algal host (Seymour et al. 2017), it was no surprise that heterotrophy was one of the most commonly detected functions amongst bacterial communities. In contrast, the prevalence of anaerobic respiration processes was unexpected. It is likely that this is a result of anaerobic conditions during the night, as the combined algal and bacterial respiration might rapidly deplete any available dissolved oxygen (Edmundson and Huesemann 2015).

The importance of the host and the environment

The variation partitioning showed that morphological and phylogenetic differences between hosts had a limited, yet significant, influence on the algal microbiomes. As evidenced by the comparison of the microbiome data to those simulated under the null model, the influence of the host, together with the isolation process, was far more extensive than could be quantified by the variation partitioning analyses (Stegen et al. 2013). The effects of the hosts on their microbiome were partially captured by the host phylogeny. This does not necessarily imply coevolution between host and microbiota (Moran and Sloan 2015), but, more likely, is a result of closely related hosts producing similar exudates (Becker et al. 2018), which in turn have similar effects on the bacterial communities. Comparable patterns has been observed for several marine organisms and their bacterial communities (e.g. fish and their skin microbiome, Chiarello et al. 2018; sponges and their microbiome, Easson & Thacker 2014), yet rarely between such closely related hosts (Swierts et al. 2018). In addition, the genetic differences between the hosts could be at the basis of the high variability in bacterial abundances. These different bacterial abundance profiles, i.e. different states, could however also be the result of ecological drift. Drift is likely to lead to the loss of bacteria in the microbiomes after extended periods of cultivation. As bacterial community dynamics were not measured in this study, it is impossible to know if that was the case.

The lower phylogenetic diversity within communities compared to the null model is a result of habitat filtering (Horner-Devine and Bohannan, 2006). As the differences between sampling locations were maintained in the null model and culture conditions were identical between communities, the only differences in ‘habitat’ are those related to the hosts. The diatoms are thus selecting closely-related bacteria in their microbiome. Remarkably, this phylogenetically limited set of bacteria is capable of performing more functions than expected under the null model. This indicates, as previously suggested (Lemanceau et al. 2017), the importance of the bacterial functions in microbiomes. A similar pattern of conserved functions in the microbiome of the macroalgae *Ulva australis* (Burke et al. 2011) led to the formulation of the competitive lottery model for microbiomes. This model encompasses both selective and neutral processes (Munday 2004). It formulates that the core of the microbiome is occupied by bacteria capable of performing the required functions. These bacteria are stochastically recruited from the bacterial source community (Burke et al. 2011). The

observed positive relation between the frequency at which bacteria were associated with their hosts and the abundance of these bacteria in the bacterial source community is in accordance which such a lottery model. This pattern also explains the large, lasting influence of the environment on microbiome as detected by the variation partitioning.

Based on our findings, the influence of the hosts and their environment on the microbiomes is clearly context dependent and will therefore be influenced by the experimental setup. The effect of the sources will be even more pronounced as more diverse habitats are sampled, due to the lottery dynamics. Similarly, more genetically distinct hosts are likely to have more dissimilar microbiomes. A stronger selection effect of the host might be promoted by reinforcing the differences between hosts or stimulating stronger interactions. The latter could potentially be achieved by increasing stress on the organisms (Maestre et al. 2005), but also by adjusting the medium in which the diatoms were cultivated. Here, the medium contained vitamins and bioavailable iron, thereby reducing the need for bacteria to provide these (Croft et al. 2005; Soria-Dengg et al. 2001). Other aspects of the cultivation conditions, such as nutrient concentrations have previously been shown to influence diatom-bacteria interactions (Grossart 1999) and are therefore also expected to have an influence.

Bacteria living together

In addition to the selection imposed by the host and the environment, the interactions between the bacteria themselves were important in structuring the microbiomes. The generally positive correlations between bacteria, the lower Bray-Curtis dissimilarity and C scores between samples all point towards the predominance of positive interactions between bacteria. Positive interactions can increase the performance of the microbiome (e.g. Ren et al. 2015) and therefore be beneficial to the host. However, they might also destabilize the microbiomes by coupling cooperative species dynamics (Coyte et al. 2015). Since co-occurring bacteria tended to be closely related, a significant overlap in resource utilization and, concurringly, intense competition between bacteria could be expected (Zelezniak et al. 2015). Performing complementary functions, as is the case based on the observed functional patterns (see above), reduces competition between the interacting bacteria and allows co-survival on the host. Processes such as cross-feeding between bacteria can further enhance positive co-occurrence patterns (Goldford et al. 2018). The effect of bacteria-

bacteria interactions on the structuring of the microbiomes warrants further research. For instance, historical events, such as priority effects (De Meester et al. 2016) could be obscuring other selectional effects.

Conclusions

In this study, we analysed and compared the relatively simple microbiomes of *C. closterium*. Host identity and the isolation process largely determined the functional and phylogenetic makeup of the algal microbiome and led to a restricted core microbiome. The imprint of the bacterial source community remained intact due to lottery dynamics in the recruitment of functionally equivalent bacteria.

The differences between the closely-related hosts induced important variation in the microbiomes, showing how selection simultaneously causes convergence (core microbiome) and divergence (variable microbiome) of the bacterial communities. The predicted competition between the bacteria was not observed, instead, positive interactions between bacteria seemed dominant, even though co-occurring bacteria were generally closely related.

The ecological framework, applied here, successfully allowed us to identify the processes underlying the observed influence of the host and the environment on the microbiome. By applying this framework to other studies and organisms, we can compare the strength of these processes and ultimately start generalising how host and environment shape the microbiome.

Acknowledgements

We thank T. Grosemans, C. Gerhard, S. De Decker and D. Zervas for their help with the sampling. The work of W. Stock was funded by the Fund for Scientific Research – Flanders (1115717N; FWO-Flanders, Belgium) and the UGent Special Research Fund (BOF).

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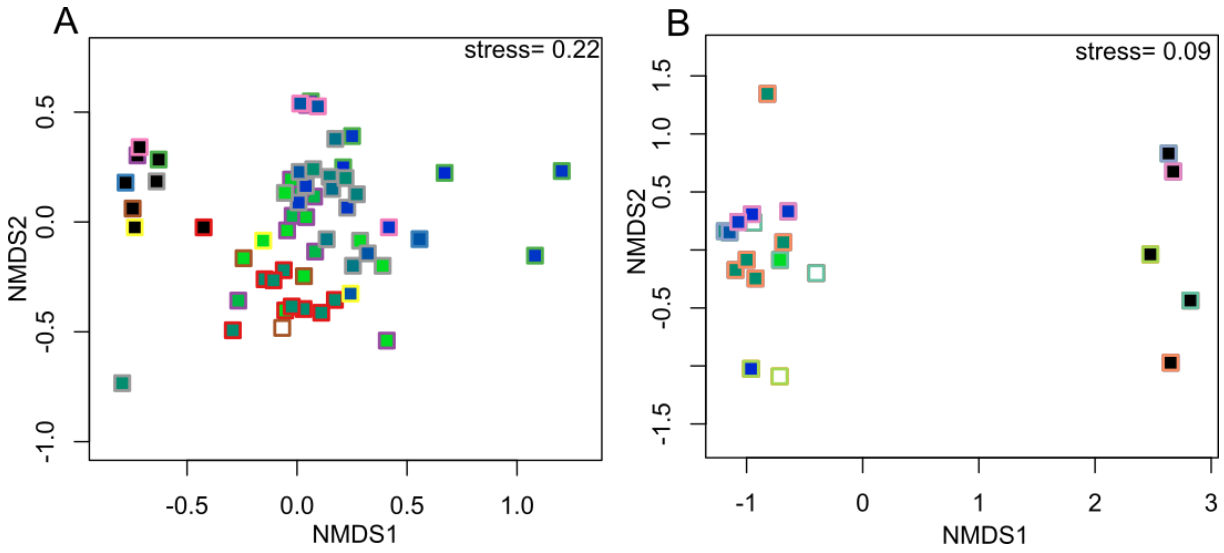
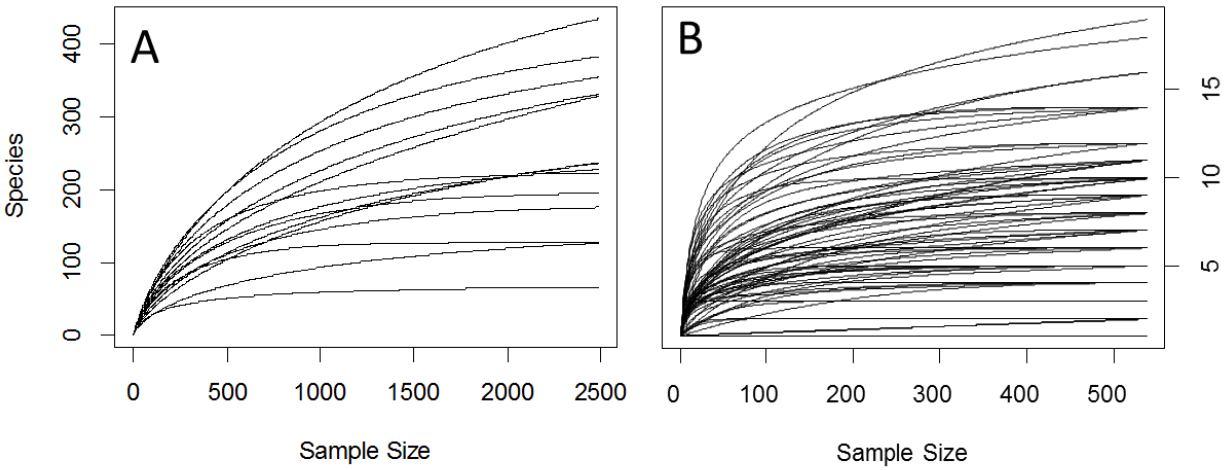
Supplementary material

Supplementary Table 1 : The variables used and retained in the variation partition analyses.

Source				Host				variables for overarching analyses
geography		environmental conditions		phylogenetic position		culture characteristics		
variables	retained variables	variables	retained variables	variables	retained variables	variables	retained variables	
spatial PCNM 1-7	PCNM 1-4	planktonic/benthic	planktonic/benthic	phylogenetic PCNM 1-23	PCNM 1, 4, 7, 12, 22	cell density	cell perimeter	source/host sample
		salinity	salinity			cell length		
		nitrite	nitrite			cell perimeter		
		nitrate	silicate					
		silicate						
		ammonia						
		phosphate						

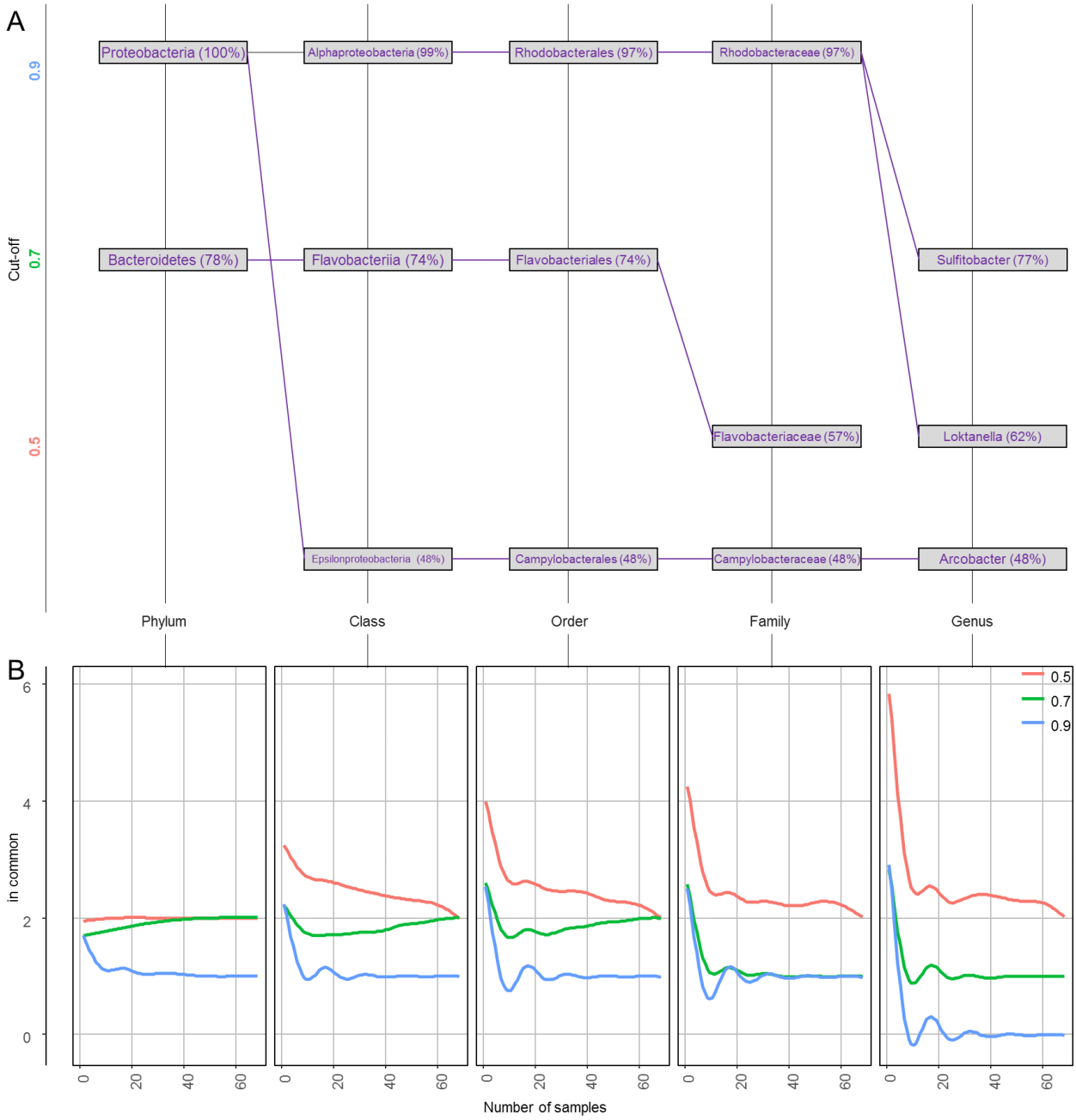


Supplementary Figure 1: Non-metric multidimensional scaling shows marked differences between the bacterial communities in the original sediment samples compared to those in the diatom cultures. The bacterial communities of both the benthic (A) and the planktonic (B) environmental samples are displayed as black squares. *C. closterium* cultures are displayed in green to blue symbols with the colour shading indicative of the genetic differences between the algal strains (genetic distance to strain CA1_1). Other diatom cultures are shown as white squares. The

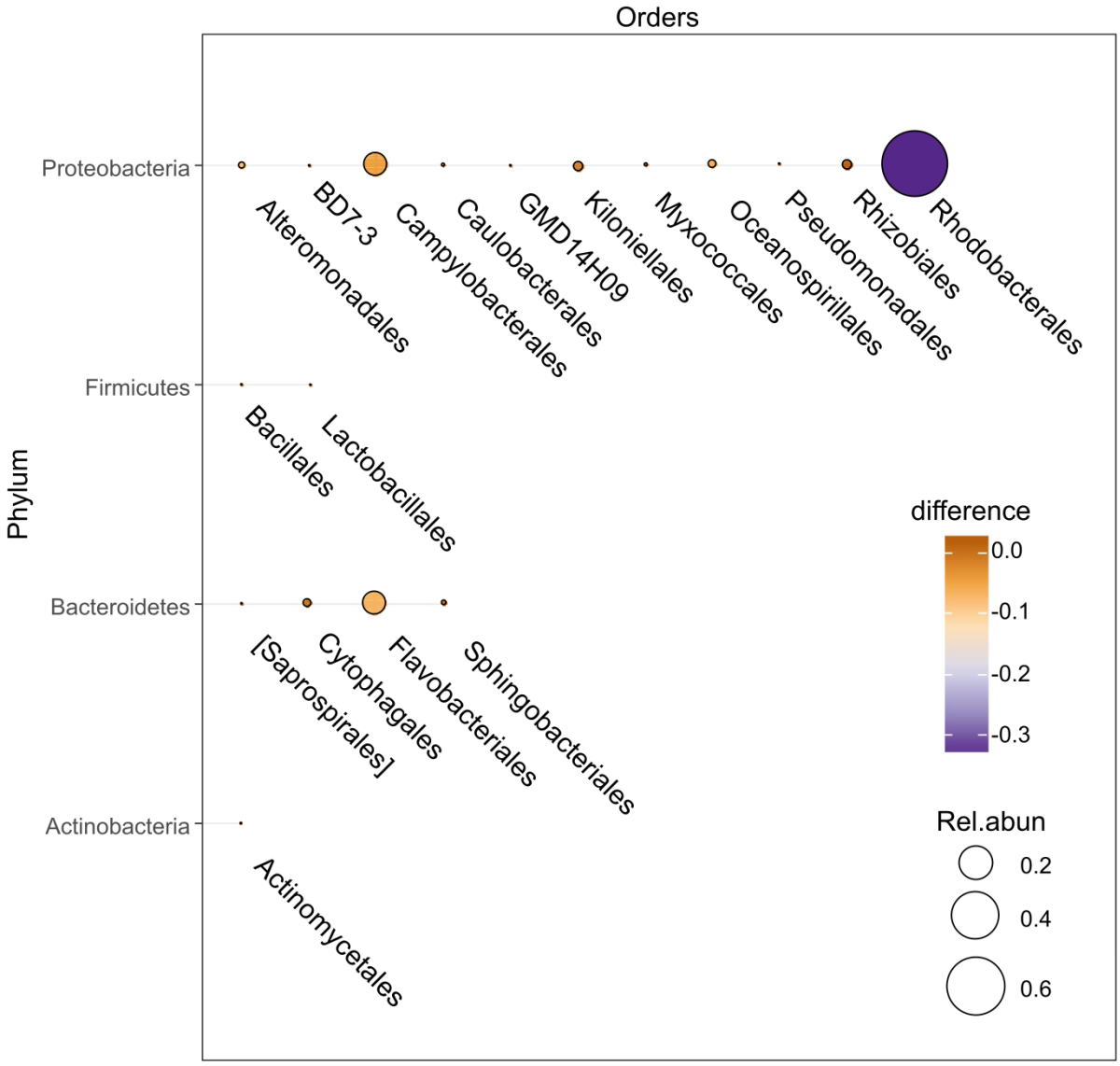


outline colours of the squares indicate the different sample locations. The two-dimensional stress is shown for each NMDS.

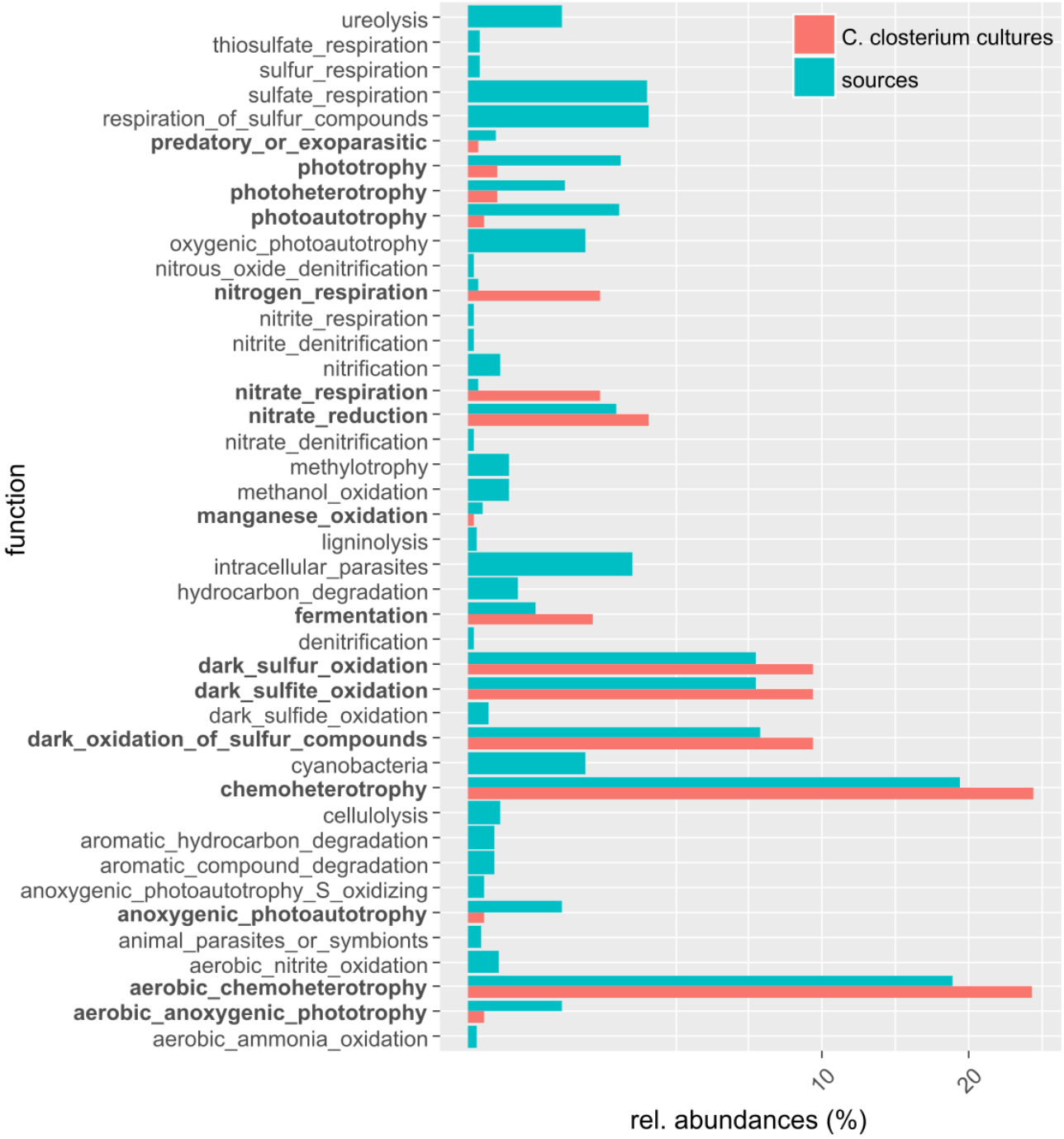
Supplementary Figure 2: rarefaction curves. The rarefaction curves of the sediment (A) and the diatoms (B) after removal of reads below four to the depth they were rarefied to for further analyses.



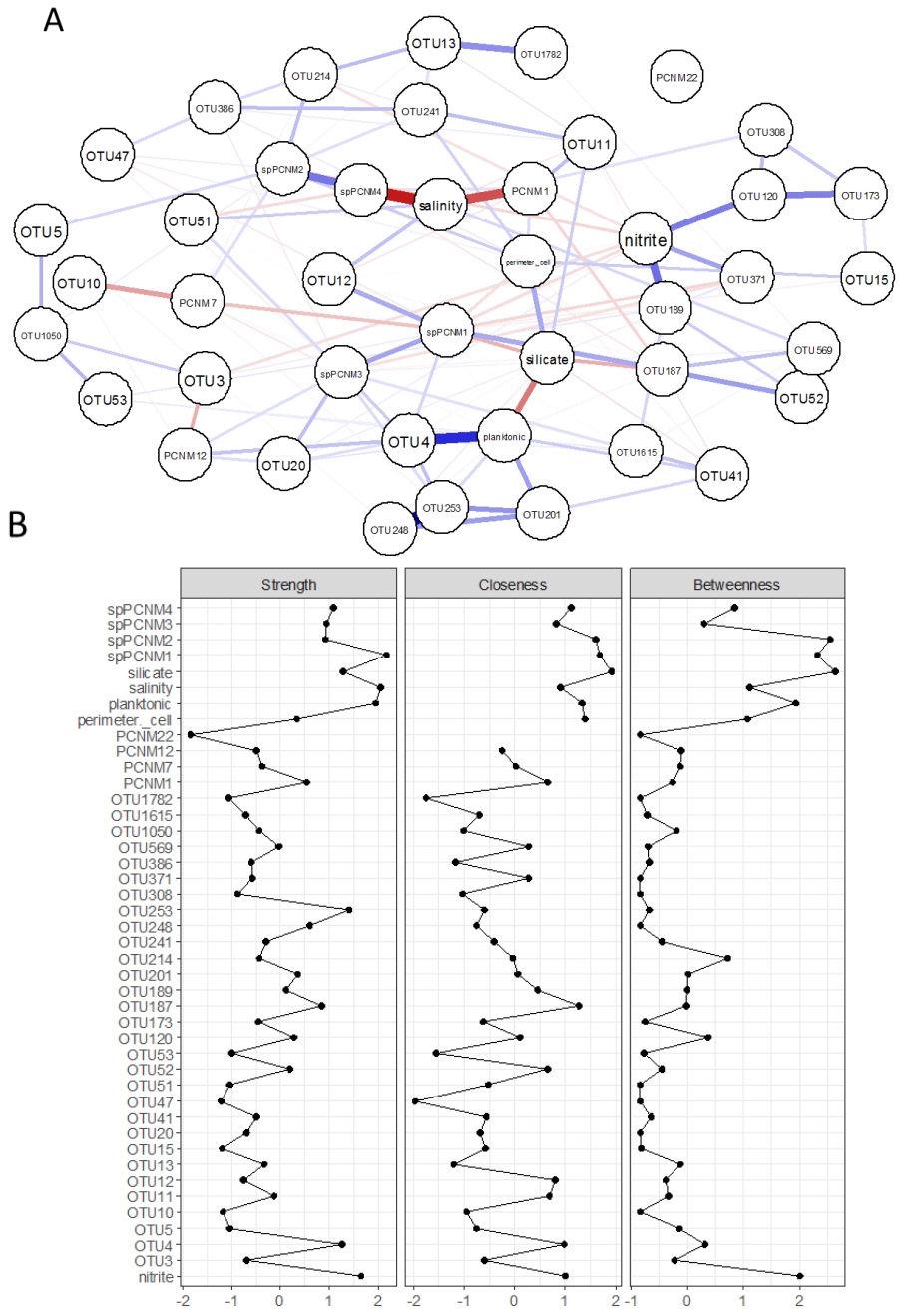
Supplementary Figure 3: most prevalent taxa in the microbiomes of *C. closterium*. The different taxonomic levels are shown from left to right in hierarchical order. (A) shows the most observed taxa with the frequency they were observed between brackets. The height of the taxa is approximate for the frequency and thus the prevalence cut-off at which they still would be observed (y-axis). The lower taxa nested within higher taxonomic levels are connected by purple lines. (B) shows the average number of taxa in common between microbiomes after repeated bootstrapping of those microbiomes. The different colours indicate the prevalence cut-offs used.



Supplementary Figure 4: Relative abundance of the bacteria in the source samples. The size of the sphere is proportional to the average relative abundance in the source samples and the colour represents the difference between the average relative abundance in sources and that in the cultures. Only the orders present in at least 2 different source samples are shown.

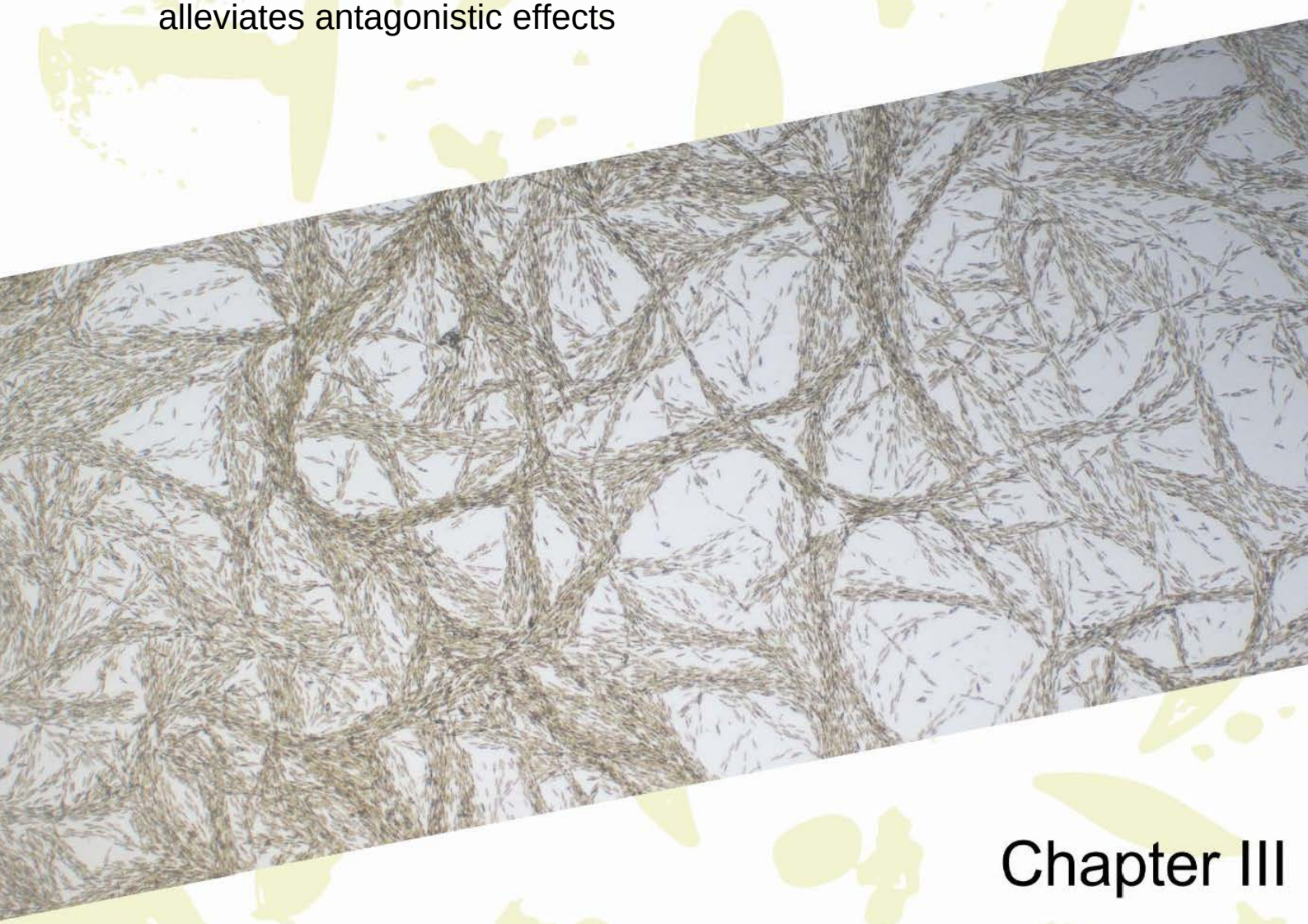


Supplementary Figure 5: the selective enrichment of functions in the *C. closterium* cultures. The average relative abundance of the functions as annotated with FAPROTAX are shown. Remark that the x-axis is log-scaled. The functions present in the algal cultures are in bold.



Supplementary Figure 6: Regularized partial correlation network with the OTUs and additional variables. (A) shows the network that was generated. The strength of the partial correlations is illustrated by the width of the edges (connections) and the colour is indicative of the sign. Remark the predominance of blue (positive) correlations between the OTUs and the more central positions of the additional variables. The strength and centrality of the variables is shown in (B). Centrality indices include closeness, the average length of the shortest path between the node and all other nodes, and betweenness, the number of times a node acts as a bridge along the shortest path between two other nodes. Bootstrapping showed that the network parameters were not stable and should thus be interpreted with care.

Chapter III: Host specificity in diatom-bacteria interactions alleviates antagonistic effects



Chapter III

Host specificity in diatom-bacteria interactions alleviates antagonistic effects

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Abstract

Microalgae live in close associations with bacteria. While different microalgae tend to be associated with different bacteria, it remains unclear if such specific associations are beneficial for the microalgae. We assessed the impact of bacterial isolates, derived from various marine benthic diatom hosts, on the growth of several closely related strains belonging to the *Cylindrotheca closterium* diatom species complex. We first tested the effect of thirty-five different bacterial isolates on the growth of a single *C. closterium* strain, and then evaluated the impact of eight of these isolates on the growth of six different *C. closterium* strains and one *C. fusiformis* strain. Surprisingly, most interactions were neutral to antagonistic; no significant positive effects of the bacteria were observed. The interactions were highly specific, with diatom growth in the presence of specific bacteria differing between *Cylindrotheca* strains and species, and closely related bacteria eliciting contrasting diatom growth responses. These differences could be partly related to the origin of the bacterial isolates, with only isolates from foreign diatom hosts being responsible for significant reductions in diatom growth, implying coadaptation between different *Cylindrotheca* strains and their associated bacteria. Interestingly, the antagonistic effect of a *Marinobacter* strain was either alleviated or enhanced by the presence of microbial inocula that were respectively native or foreign to the diatom host, suggesting coadapted bacteria might also benefit their host indirectly by preventing the establishment of harmful bacteria.

Introduction

The coexistence of heterotrophic bacteria and microalgae over evolutionary times has fostered intimate associations between them. Such microscale associations should come as no surprise, as bacteria directly mineralize a large fraction of the dissolved and particulate organic matter produced by microalgae to inorganic compounds, part of which can then be reutilized by the microalgae (Amin et al., 2012; Buchan et al., 2014; Mühlenbruch et al., 2018). During the past decades other types of interactions, ranging from antagonistic to mutualistic, have also been documented (Amin et al., 2012; Kouzuma and Watanabe, 2015; Meyer et al. 2017; Ramanan et al., 2016). Some heterotrophic bacteria support algal growth by supplying essential growth factors, such as vitamin B12 and auxins (Amin et al., 2015; Croft et al., 2005), while others exert antagonistic bioactivity by producing algicides (Paul and Pohnert, 2011) or by interfering with cellular signaling (Cirri et al., 2018). The outcome of algal-bacterial interactions can be modified by abiotic or biotic cues, resulting in sometimes highly dynamic interactions (Seyedsayamdost et al., 2011b). Prompted by the right signals, such as direct contact with the algal cell, bacteria might suppress or activate algicidal pathways (Skerratt et al., 2002; Wang et al., 2014).

Only a few bacterial phyla are commonly found in association with algae (Jasti et al., 2005; Rizzo et al., 2016; Singh and Reddy, 2014) including Proteobacteria (mainly Alpha- and Gammaproteobacteria) and Bacteroidetes (especially Flavobacteriia). The same groups also commonly associate with diatoms (Amin et al., 2012), a group of unicellular stramenopile algae responsible for about one fifth of the global primary production (Yool and Tyrrell, 2003). Several studies have demonstrated close associations between diatoms and bacterial taxa (Behringer et al., 2018; Grossart et al., 2005; Schäfer et al., 2002) and even closely-related diatom strains were shown to harbour distinct bacterial communities (Sison-Mangus et al., 2014). Furthermore, by observations based on bacterial transplant experiments, Sison-Mangus and colleagues (2014) showed that bacteria can be mutualistic to their native host but commensal or even antagonistic towards a foreign host, hinting at co-evolution between bacteria and their host. Other studies however suggest that diatom-bacteria associations are more random, not determined by evolutionary processes (Meyer et al., 2017), and variable in space and time. For example, Ajani et al. (2018) reported a strong environmental imprint on the bacterial communities associated with various

Leptocylindrus spp., and Sapp et al. (2007) reported pronounced compositional shifts during cultivation, suggesting no specificity in the diatom-bacteria associations. Likewise, a strain of *Pseudo-nitzschia multiseries* developed a completely different bacterial community when transferred to another lab (Kaczmarska et al., 2005). As it was shown that even *Escherichia coli* laboratory strains can enhance diatom growth and alter diatom behaviour (Bruckner et al., 2011), it appears that coadaptation between bacteria and their host does not seem to be a prerequisite for effective interactions between them.

In this study we focussed on interactions between marine benthic diatoms and their associated bacteria. In tidal flats, both groups of microorganisms co-occur in biofilms, in much denser and more closely packed associations than in pelagic systems, which have been the subject of most algae-bacterial interaction studies to date (Amin et al., 2012; Ramanan et al., 2016). These biofilms are essential for the functioning of the tidal flat ecosystem by regulating nutrient and carbon fluxes, stabilizing sediments and fuelling coastal food webs (Van Colen et al., 2014). Diatom and bacterial activities are tightly coupled in these biofilms, as is evidenced by the rapid carbon flow from diatom photosynthate to bacteria (Middelburg et al., 2000) and the strongly correlated dynamics of both communities (Agogué et al., 2014; Decleyre et al., 2015; Moerdijk-Poortvliet et al., 2014; Taylor et al., 2013).

The general aim of this study was to assess whether interactions between benthic diatoms and their associated bacteria were species- and strain-specific or not. To this end, we tested the effects of thirty-five diatom-derived bacterial isolates on the growth rate of six strains belonging to the *Cylindrotheca closterium* (Ehrenberg) Reimann & Lewin species complex (Stock et al., 2019b; Vanormelingen et al., 2013) and one *C. fusiformis* Reimann & Lewin strain. *Cylindrotheca* is an ecologically important diatom genus which is abundant in marine and brackish benthic habitats worldwide (De Round et al. 1990; Stock et al., 2019b). We hypothesized that reciprocal evolutionary interactions between the hosts and their microbiomes has led to coadaptation between both, leading to a high level of specificity in the interactions. We therefore expected bacteria to stimulate the growth of their native host but not the growth of a foreign host, that closely related algal hosts would respond in a similar fashion towards bacteria, and that closely related bacteria would elicit similar growth responses in the diatoms. In addition, as diatom-bacteria interactions naturally take place in a highly complex

biological and environmental context, which can impact the effect that interaction partners have on each other (Mayali and Doucette, 2002; Roth et al., 2008), we tested if specific effects of bacteria on diatom growth could be modulated by the composition of the background bacterial community.

Materials and methods

Diatom isolation, culturing, genetic identity and phylogeny

Six *Cylindrotheca closterium* strains (WS_{3_7}, OS_{4_13}, SO_{3_2}, NH_{3_12}, CA_{1_8}, ZW_{2_4}) and one *C. fusiformis* strain (IIDO2) were isolated by micropipetting (Andersen et al. 2005) from six different estuarine sites in NW Europe (Supplementary Table 1). Diatoms were maintained in the lab at 18°C and 12L:12D light regime (25 µmol photons m^{-2} s^{-1}cool fluorescent TL light) in autoclaved Artificial Sea Water (ASW) (Tropic Marine Bio-Actif Salt) supplemented with filter-sterilized (Acro-disc Syringe Filter, 0.2µm) Guillard's F/2 solution (Sigma Aldrich, G0154). The same medium (ASW+F/2) was used for all experiments described below.

DNA was extracted from subsampled diatom cultures following Zwart et al. (1998) using a bead-beating method with phenol. *RbcL* and ITS1 sequences were obtained as described in Vanelslander et al. (2009). Sequences were aligned using ClustalW and afterwards manually curated in Mega 7 (Kumar et al., 2016). Phylogenetic relationship was inferred based on the concatenated *rbc*L and ITS1 alignments via maximum likelihood (ML) and Bayesian inference (BI). Partition Finder 2 (Lanfear et al., 2016) was used for selecting best-fit partitioning schemes and models of evolution for nucleotide substations. RaxML (Stamatakis, 2006) was used for ML based phylogenetic inference using a four subset partition model (1 for ITS1 and 3 for the different codon positions of *rbc*L) and *C. fusiformis* (strain IIDO2) as outgroup. Mr Bayes (version 3; Ronquist and Huelsenbeck, 2003) was used for Bayesian inference using the same partition model. Two independent runs of four Metropolis-coupled Monte Carlo Markov Chains were run for 3 million generations and sampled every 100^{th} generation. After inspection, the first 25% of the samples were discarded after which a consensus tree was generated using the remaining samples.

An additional ML-based phylogeny was constructed using *rbc*L sequences of representatives of the different clades in the *C. closterium* complex as delineated by

Vanormelingen et al. (2013) (viz. PS2, OS1, OS13 & OS9B) and the strains used in this study. A substitution model was used in which the 3 different codon positions of *rbc*L were defined, after which the data were analyzed as described above.

Isolation and identification of diatom-associated bacteria

Within four months after isolating the diatoms, heterotrophic bacteria were isolated from the six *Cylindrotheca closterium* cultures using two types of solid media: agar [5.6 L^{-1} Marine Agar (Difco) and 15 gL^{-1} Bacto Agar (Difco)] enriched with either 4g L^{-1} glucose or 10% diatom spent medium, collected from dense, late exponential to stationary *C. closterium* cultures. In addition, bacteria were isolated from strains of the benthic diatoms *Seminavis robusta* and *Cylindrotheca fusiformis* obtained from the BCCM/DCG culture collection (http://bccm.belspo.be/about-us/bccm-dcg; *S. robusta* 85A and *C. fusiformis* IIP03, IID02 and W02-22) using Difco Marine Agar and Difco Marine Agar enriched with 2 gL^{-1} glucose and 2 gL^{-1} galactose. For isolation, part of the diatom cultures was spread out over an agar plate after which they were incubated at 20°C. Colonies were selected based on their unique morphology and purified by repeated plating on Difco Marine Agar. Each strain was given a unique identifier: the first two letters refer to genus and species name of the host diatom, followed by a two-letter abbreviation of the geographical origin of the host (Table 1).

Bacterial isolates were identified by partial sequencing of 16S rDNA as described in Edwards et al. (1989). Sequences were aligned and verified using BioNumerics 5.1 (Applied Maths, Belgium). Identifications were done using the RDP classifier tool (Wang et al., 2007).

Table 1: List of bacterial isolates. For identifier, see text and supplementary table 1. R code: research collection number of the non-public part of the BCCM/LMG Bacteria Collection (http://bccm.belspo.be/about-us/bccm-lmg). Isolation medium: medium of original inoculation. *MA*: marine agar (Difco), in some cases enriched with glucose (*Glu*), glucose and galactose (*Glu&Gal*) or spent medium from a *Cylindrotheca closterium* culture (*spent*). Classification based on partial 16S sequence similarity (see text). Experiment columns indicate in which experiment a particular strain was used.

R Code	Class	Family	Genus	Identifier	Source	Isolation Medium	exp. 1	exp. 2	exp. 3
R-54394	Actinobacteria	Micrococcaceae	*Arthrobacter*	CcWSa	*Cylindrotheca closterium*	MA + Glu	•	•	
R-54259	Actinobacteria	Micrococcaceae	*Arthrobacter*	CcWSb	*Cylindrotheca closterium*	MA + Glu	•		
R-54395	Actinobacteria	Nocardioidaceae	*Nocardioides*	CcWSc	*Cylindrotheca closterium*	MA + Glu	•		
R-54271	Alphaproteobacteria	Moraxellaceae	*Acinetobacter*	CcZWa	*Cylindrotheca closterium*	MA + Glu	•		
R-54390	Alphaproteobacteria	Rhodobacteraceae	*Celeribacter*	CcSOc	*Cylindrotheca closterium*	MA + spent	•		
R-54258	Alphaproteobacteria	Rhodobacteraceae	*Celeribacter*	CcWSd	*Cylindrotheca closterium*	MA + Glu	•		
R-54267	Alphaproteobacteria	Rhodobacteraceae	*Loktanella*	CcZWc	*Cylindrotheca closterium*	MA + spent	•	•	
R-50234	Alphaproteobacteria	Rhodobacteraceae	*Phaeobacter*	CfWS2c	*Cylindrotheca fusiformis*	MA	•		
R-50241	Alphaproteobacteria	Rhodobacteraceae	*Roseovarius*	SrVMb	*Seminavis robusta 85A*	MA	•		
R-50236	Alphaproteobacteria	Rhodobacteraceae	*Ruegeria*	CfWS2e	*Cylindrotheca fusiformis*	MA	•		
R-50235	Alphaproteobacteria	Rhodobacteraceae	*Sagitulla*	CfWS2d	*Cylindrotheca fusiformis*	MA	•	•	
R-46775	Alphaproteobacteria	Rhodobacteraceae	*Stappia*	CfNSa	*Cylindrotheca fusiformis*	MA	•		
R-54388	Alphaproteobacteria	Rhodobacteraceae	*Sulfitobacter*	CcOSa	*Cylindrotheca closterium*	MA + spent	•		
R-50315	Flavobacteriia	Flavobacteriaceae	*Croceibacter*	SrVMc	*Seminavis robusta 85A*	MA	•	•	
R-46770	Flavobacteriia	Flavobacteriaceae	*Leeuwenhoekiella*	CfWS1a	*Cylindrotheca fusiformis II*	MA + Glu&Gal	•	•	
R-50232	Flavobacteriia	Flavobacteriaceae	*Leeuwenhoekiella*	CfWS2b	*Cylindrotheca fusiformis*	MA	•		
R-50239	Flavobacteriia	Flavobacteriaceae	*Maribacter*	SrVMa	*Seminavis robusta 85A*	MA	•		
R-54265	Gammaproteobacteria	Alteromonadaceae	*Alteromonas*	CcNHc	*Cylindrotheca closterium*	MA + spent	•		
R-54393	Gammaproteobacteria	Alteromonadaceae	*Alteromonas*	CcOSc	*Cylindrotheca closterium*	MA + spent	•		
R-54396	Gammaproteobacteria	Alteromonadaceae	*Alteromonas*	CcZWd	*Cylindrotheca closterium*	MA + spent	•		
R-50228	Gammaproteobacteria	Alteromonadaceae	*Alteromonas*	CfWS2a	*Cylindrotheca fusiformis*	MA	•		
R-46755	Gammaproteobacteria	Alteromonadaceae	*Marinobacter*	SrVM1a	*Seminavis robusta 85A*	MA	•	•	
R-46757	Gammaproteobacteria	Alteromonadaceae	*Marinobacter*	SrVM1b	*Seminavis robusta 85A*	MA	•		
R-50317	Gammaproteobacteria	Alteromonadaceae	*Marinobacter*	SrVMd	*Seminavis robusta 85A*	MA	•		
R-50319	Gammaproteobacteria	Alteromonadaceae	*Marinobacter*	SrVMe	*Seminavis robusta 85A*	MA	•	•	•
R-54262	Gammaproteobacteria	Alteromonadaceae	*Marinobacterium*	CcCAd	*Cylindrotheca closterium CA*	MA + spent	•		
R-46777	Gammaproteobacteria	Alteromonadaceae	*Paraglaciecola*	CfNSb	*Cylindrotheca fusiformis*	MA + Glu&Gal	•		
R-54389	Gammaproteobacteria	Oceanospirillaceae	*Amphritea*	CcSOa	*Cylindrotheca closterium*	MA + spent	•	•	
R-54256	Gammaproteobacteria	Oceanospirillaceae	*Marinomonas*	CcOSb	*Cylindrotheca closterium*	MA + spent	•		
R-54386	Gammaproteobacteria	Pseudoalteromonadaceae	*Pseudoalteromonas*	CcCAb	*Cylindrotheca closterium CA*	MA + Glu	•		
R-54264	Gammaproteobacteria	Pseudoalteromonadaceae	*Pseudoalteromonas*	CcNHa	*Cylindrotheca closterium*	MA + Glu	•		
R-54257	Gammaproteobacteria	Pseudoalteromonadaceae	*Pseudoalteromonas*	CcNHb	*Cylindrotheca closterium*	MA + spent	•		
R-54385	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*	CcCAa	*Cylindrotheca closterium CA*	MA + Glu	•		
R-54392	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*	CcWSf	*Cylindrotheca closterium*	MA + spent	•		
R-46778	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*	CfNSc	*Cylindrotheca fusiformis*	MA + Glu&Gal	•		

Axenisation of diatom cultures

To obtain bacteria-free (axenic) diatom cultures, cells were suspended and sonicated (20% intensity, 1-second interval) for 1 minute to break up cell clusters. The cultures were subsequently treated with 500 µg/mL streptomycin sulfate (Sigma Aldrich, S-9137), 100µg/mL gentamycin sulfate salt (Sigma Aldrich, G3632-16) and 100 µg/mL neomycin (Sigma Aldrich, N1142). After approximately 3 days, a second antibiotic treatment was administered using 500 µg/mL penicillin G sodium salt (Sigma Aldrich, P3032-10MU), 100 µg/mL gentamycin and 100 µg/mL streptomycin sulfate salt. This process was repeated. Prior to the experiments, diatoms were cultured in medium without antibiotics. Axenicity was confirmed by plating (Difco Marine Agar 2216, BD) and DAPI staining (Shishlyannikov et al., 2011). Stained samples were examined under an epi-fluorescence microscope (Axiophot2, Zeiss). Diatom growth was not affected by the axenisation procedure (data not shown).

Diatom-bacteria bioassays

Different co-culture experiments (see below) of diatoms and bacteria were set up in 24-well plates (Greiner CELLSTAR, Sigma-Aldrich). Diatom growth was monitored daily using the Imaging-PAM fluorometer (M-series, Walz), with dark-adapted minimal fluorescence (F_0) being used as a proxy for diatom biomass (Stock et al., 2019a).

Diatom cultures were axenic and in exponential growth phase prior to the experiments. The growth medium from these diatom cultures was refreshed and diluted to obtain a minimal fluorescence reading (F_0) of 0.05 arbitrary fluorescence units (PAM settings: measuring intensity 3, frequency 1, gain 1; approximately $4.5x10^4$ cells per well). Bacteria were collected and suspended in ASW+F/2 three days after being inoculated on Difco Marine Agar. Bacterial densities were determined using optical density at 595nm (OD_{595}) measured with a VICTOR 3 multilabel plate reader (Perkin Elmer). Bacteria were diluted to OD_{595} of 0.1 (approximately 10^8 -10^9 cells per ml). The bacterial suspensions and diatom cultures were dispensed in the well plates. The final volume in every well was always 2 ml. Experiment 1 (see below) was run in quadruplicate and experiments 2 and 3 in triplicate. All treatments were randomly distributed across the plates. Axenic controls were included in all experiments.

Coculture plates were maintained for 7 days at 18°C in a 12L:12D light regime (25 µmol photons/m/s cool fluorescent TL light). Diatom growth was inferred by daily

measurements of *in vivo* minimum fluorescence yield (F_0) using the same PAM settings as described above. Viability and bacterial contamination (in case of the axenic cultures) of the diatom cultures were daily assessed by microscopy (Zeiss Axiovert) .

In experiment 1, 35 different bacterial isolates (Table 1) were added to axenic cultures of *C. closterium* WS_{3_7} and diatom growth rate was measured (see below).

For experiment 2, eight bacterial isolates (Table 1) were crosswise added to the six *C. closterium* strains and the *C. fusiformis* strain and the diatom growth rates were measured. The eight bacterial isolates were selected in order to have representatives from all major bacterial groups and from different diatom hosts. Strains with a strong impact on diatom growth (based on the findings of experiment 1) were also included.

In experiment 3, axenic *C. closterium* WS_{3_7} was cultured with the growth inhibiting *Marinobacter* strain SrVMe and different natural bacterial inocula to assess the effect of the background bacteria community on the previously observed interaction between WS_{3_7} and SrVMe. These bacterial inocula were extracted from either the native habitat of *C. closterium* WS_{3_7}, viz. intertidal sediment, or from a foreign habitat (forest soil). Intertidal sediment from the Westerschelde estuary (N 51°21’ 1,92”; E 3°43’34,44”) was sampled during low tide. The upper few millimeters of the sediment were transferred to sterile 50 mL falcons and filled with local seawater. Forest soil was collected in Ghent, Belgium (N 51°01’27”; E 3°42’42”). The upper few millimeters were transferred to 5 sterile 50 mL falcons and filled with ASW-F/2. Bacteria were separated from the sediments by repeated vortexing followed by centrifugation (800 RCF, 8min) (Sigma, 4K15) after which the pellet (with the sediment) was discarded. The absence of eukaryotic cells was confirmed under the inverted microscope. One hundred µl of the intertidal (I) or forest (F) inoculum were added to the wells, which either contained only *C. closterium* WS_{3_7} or *C. closterium* WS_{3_7} with SrVMe. After 7 days, 1 mL of the culture samples was taken to compare the bacterial communities. DNA was extracted as described above for the diatom cultures. Amplification and Denaturing Gradient Gel Electrophoresis (DGGE) were run as described in Muyzer et al. (1993). The UV photographed gels were processed in Bionumerics 7 (Applied Maths, Belgium). Bands coinciding with the diatom chloroplast were removed from subsequent analysis.

Data analysis and statistics

For all experiments, exponential growth rates were calculated as the slope of the log2 transformed F_0 data between day 1 (the day of inoculation) and day 3 after inoculation. The cultures were not compared beyond day 3 as clustering of cells, which occurred from then onwards, and a potential decrease in fluorescence as cultures become stationary, could bias fluorometry-based biomass assessment (Stock et al., 2019a). For all analyses the statistical software package R (version 3.4.1) was used.

For experiment 1, homogeneity of variance and normal distribution of the growth rates were verified after which treatments were compared using One-Way ANOVA, followed by post-hoc analysis using Dunnett's-test (multcomp package version 1.4-8) to compare the growth of every coculture to the axenic control. To test for proportional differences in significance between bacterial origin (diatom they were originally isolated from), the odds ratios were calculated (fmsb package version 0.6.3) between the different groups.

For experiment 2, we correlated diatom phylogeny with their growth responses when exposed to the different bacteria. The growth of diatoms exposed to the bacteria was averaged over the replicates and divided by the averaged growth of that same strain under axenic conditions (the control) to obtain the growth ratio. The Euclidean distances between the diatom strains were calculated based on these ratios. On that distance matrix, a hierarchical cluster (complete linkage) analysis was performed.

The consensus maximum likelihood phylogeny was adjusted by setting all zero branch lengths to 0.00001 to avoid problems in the downstream analyses. The tree was thereafter converted into an ultrametric tree using a semi-parametric method based on penalized likelihood (λ=0.1; Sanderson, 2002). Phylogeny and the growth ratios were compared with each other by means of K_{mult} (phylocurve version 2.0.9; Adams, 2014). This parameter estimates the phylogenetic signal present in the ratios by means of stimulating the evolution of the diatom responses under Brownian motion. A mantel test (9999 permutations, *ade4* version 1.7-11) was performed on the distance matrices constructed from the growth ratios and the phylogeny.

An Abouheif's test (Abouheif, 1999) was performed to test the phylogenetic signal in the growth responses for each of the bacterial strains (adephylo version 1.1). This is

a particular form of the Moran's I test which detects phylogenetic autocorrelation (Pavoine et al., 2008) for different traits simultaneously.

Results

Identity and phylogeny of *Cylindrotheca* strains

The *C. closterium* strains displayed relatively high genetic diversity, except for SO_{3_2} and OS_{4_13}, which were identical for the sequenced regions (Supplementary Figure 1a). Two clusters can be distinguished: CA_{1_8}, SO_{3_2} and OS_{4_13} versus ZW_{2_4}, NH_{3_12} and WS_{3_7}. The position of ZW_{2_4} however is poorly supported: it is either sister to WS_{3_7}/NH_{3_12} (ML consensus tree) or to all other *C. closterium* strains (BI consensus tree, not shown). All other bifurcations in the tree are well supported. No relationship between geographical origin and phylogenetic topology was observed.

The observed clades agree well with those previously defined by Vanormelingen et al. (2013) and Stock et al. (2019b). NH_{3_12}/WS_{3_7} belong to clade V *sensu* Vanormelingen et al. (2013), which was delineated as putative species 1 in Stock et al. (2019b). ZW_{2_4} belongs to clade IV *sensu* Vanormelingen et al. (2013), delineated as putative species 2 in Stock et al. (2019b). The other three strains form a distinct cluster in between clades IV/V and clade III *sensu* Vanormelingen et al. (2013); strains OS_{4_13} and SO_{3_2} belong to putative species 4 of Stock et al. (2019b). It is most likely that these different clades represent different species within the *C. closterium* species complex (Vanormelingen et al. 2013, Stock et al. 2019b).

Identity of bacterial isolates

Sequencing of partial 16S rDNA revealed high bacterial taxonomic diversity (Table 1). All sequences showed high to very high similarity (99-100%) with RPD database reference sequences. The isolates mainly belonged to Alpha- and Gammaproteobacteria and to a lesser extent to Actinobacteria and Flavobacteriia. Three genera were retrieved from more than one *C. closterium* strain: *Celeribacter* (CcSOc & CcWSd), *Pseudoalteromonas* (CcCAb, CcCAb, CcNHa & CcNHb) and *Alteromonas* (CcNHc, CcZWd & CcOSc). All other bacterial isolates from the *C. closterium* cultures were, at the genus level, unique to one of the *C. closterium* cultures.

Experiment 1: Impact of bacteria on growth dynamics of *C. closterium* WS_{3_7}

Out of the 35 bacterial isolates tested on *C. closterium* WS_{3_7}, nine resulted in a mean diatom growth rate higher than the axenic control (Figure 1). The other 26 isolates

reduced the growth rate compared to the control, with six isolates significantly reducing the growth rate. The latter belonged to the Flavobacteria (*Croceibacter* and *Leeuwenhoekiella*) and Gammaproteobacteria (*Alteromonas* and *Marinobacter*). None of the significantly growth-inhibiting bacteria was isolated from *C. closterium* WS_{3_7}, the strain used in this assay. Only one of the bacteria was isolated from another *C. closterium* strain (CcOSc), assigned to different putative *C. closterium* species than WS_{3_7}, was significantly growth inhibiting; two other growth-inhibiting bacteria were isolated from *C. fusiformis* and three from *S. robusta*. A significantly ($p=0.045$) higher proportion (31%) of bacterial strains isolated from other diatom species (*C. fusiformis* and *S. robusta*) thus turned out to inhibit the growth of *C. closterium* WS_{3_7} in comparison to only 4% of the bacteria isolated from *C. closterium* strains, despite the fact that most strains were isolated from the latter.

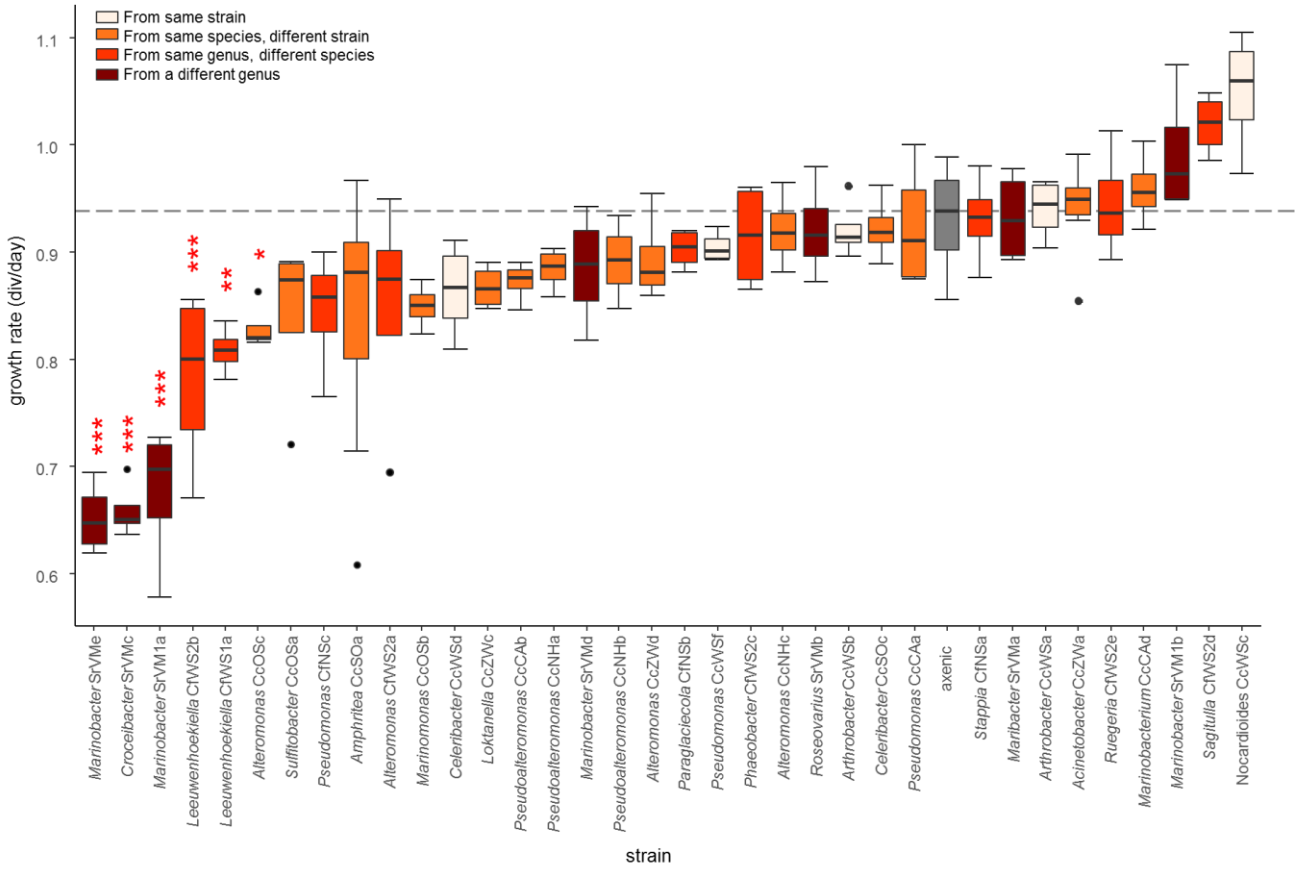

Figure 1: Growth rates of *C. closterium* WS_{3_7} in coculture with 35 different bacterial isolates. The growth of the diatom, expressed as divisions per day on the y-axis, is mostly lower in the presence of bacteria compared to the growth under axenic conditions (grey box, grey dashed lined indicates median growth of the axenic control). The bacterial treatments are sorted from low to high mean growth rate. Colours of the boxes indicate the relationship between *C. closterium* WS_{3_7} and the diatom host the bacteria were originally isolated from. Significant differences in growth rate compared to the axenic control are indicated with red asterisks (***p < 0.001; ** 0.001 < p < 0.01; * 0.01 < p < 0.05).

Experiment 2: Strain-specificity in diatom growth response towards bacteria

A selection of 8 bacteria was cocultured with the 6 different *C. closterium* strains and the single *C. fusiformis* strain. Axenic diatom growth rates ranged from 0.8 to 1 divisions/day. The bacterial impact on the growth rate of *C. closterium* WS_{3_7} was comparable to the previous experiment, except for the *Croceibacter* strain SrVMc which lost its growth inhibiting effect and the *Arthrobacter* strain CcWSa which switched from slightly positive to negative. Both switches were confirmed by additional growth experiments (data not shown). For all diatom strains, both stimulation and

inhibition of growth by the bacteria were detected (Figure 2), with growth ratios ranging between 0.16 and 1.4. A majority (32 out of the 56) of the diatom-bacteria combinations had a lower growth than the axenic controls. All bacteria, expect the *Marinobacter* strain SrVMe, could induce both growth enhancement and inhibition, depending on the diatom strain. SrVMe induced pronounced growth inhibition in all tested diatom strains. In all cases, microscopic inspection revealed that growth inhibition by this strain was a result of the inhibition of cell division, rather than cell lysis.

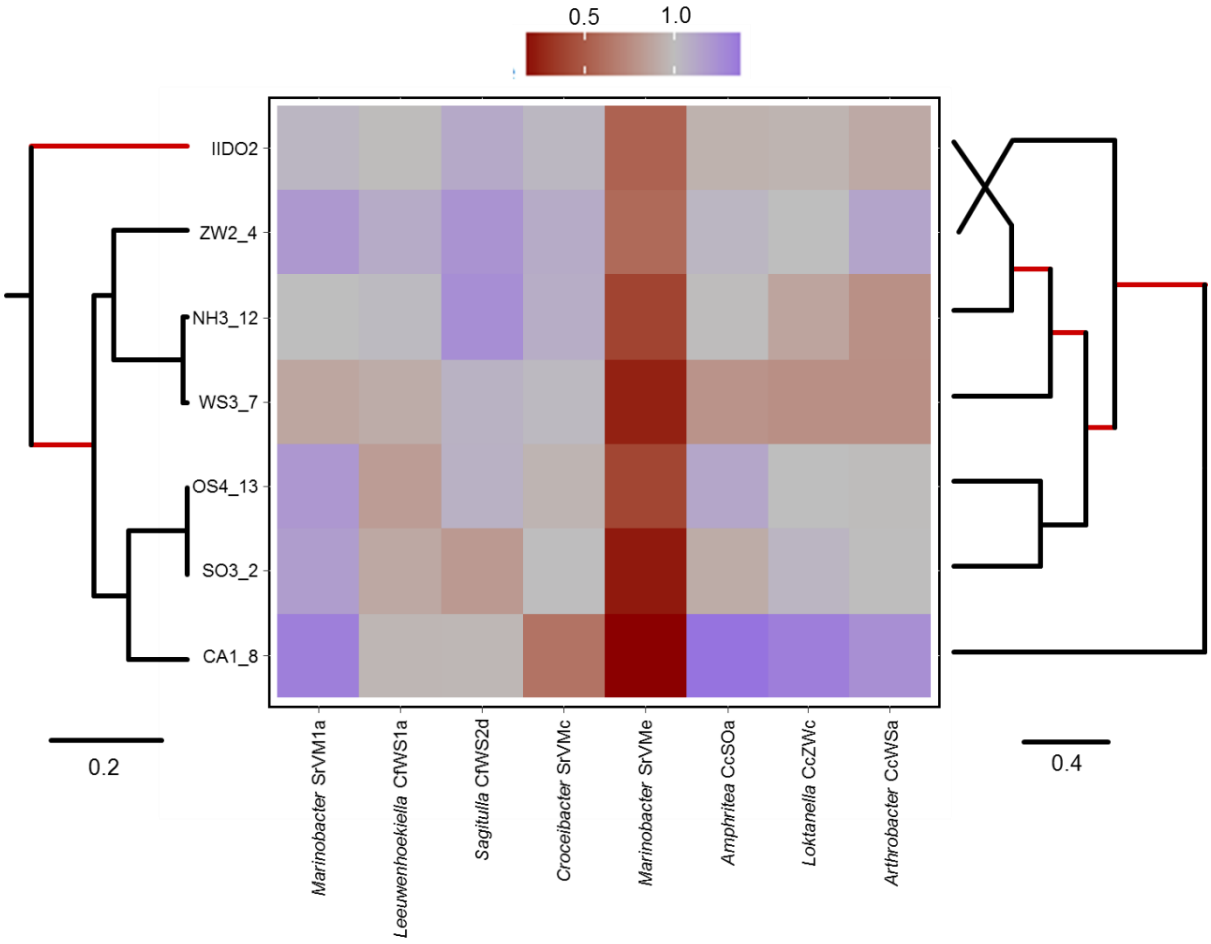

Figure 2: Strain-specific growth responses of 6 *Cylindrotheca closterium* and 1 *C. fusiformis strains* in cocultures with 8 different bacteria. The growth responses (compared with the axenic control) of the diatom strains (rows) is shown as a heat map. Unaltered growth is indicated in grey; growth inhibition is displayed in red and growth enhancement in blue. The *Cylindrotheca* phylogeny (maximum likelihood consensus tree, see supplementary figure 1 for more details) is shown on the left of the heat map. On the right, a hierarchical cluster analysis based on the diatom growth responses is shown. Branches which have been shortened to allow a better visualisation of both cladograms are indicated by red lines.

The diatom strains displayed different growth responses in the presence of bacteria. Some strains (including the *C. fusiformis* strain IIDO2) were more similar in their responses (Figure 3, cladogram on the right), namely IIDO2, WS_{3_7} & NH_{3_12} and SO_{3_2} & OS_{4_13}. In contrast, CA_{1_8} and ZW_{2_4} had quite distinct responses to the bacteria. There was however no congruence between the phylogeny of the hosts and their growth response. K_{mult}, which estimates the phylogenetic signal in the diatom growth response, was extremely small (0.0001) and not significant ($p>0.05$), nor was the Mantel test ($p>0.05$) which tested the correlation between phylogenetic distances and the distances based on the diatom growth responses. Abouheif's test, based on Moran's I and designed to detect phylogenetic autocorrelation, was not significant ($p>0.05$) for any of the bacteria either.

Experiment 3: effect of background bacteria on the diatom-bacteria interaction

C. closterium WS_{3_7} was cocultivated with the strongly growth inhibiting *Marinobacter* strain SrVMe. To test the effect of the presence of other bacteria on this interaction, natural bacterial inocula, extracted from an intertidal sediment and a forest soil, were added to the coculture. SrVMe strongly inhibited growth when cultured alone with the diatom (Figure 3). Addition of the intertidal or forest inocula to the diatom culture did not affect diatom growth when compared to the axenic control. The addition of both the *Marinobacter* strain and the forest inoculum resulted in an even stronger growth inhibition than in the coculture with the *Marinobacter* strain alone. In contrast, when both the *Marinobacter* strain and the intertidal inoculum were added to the diatom culture, diatom growth recovered by the fourth day, and after 7 days diatom densities were comparable to those in the axenic control. DGGE analysis (Figure 3) revealed that in the *Marinobacter* + forest inoculum treatment, the *Marinobacter* strain could still be detected in all replicates, representing about 44% of the total band intensity. In contrast, no *Marinobacter* band could be detected in two out of the three replicates of the diatom cultures inoculated with *Marinobacter* and the intertidal inoculum. In the single replicate which did still contain *Marinobacter*, its band had a relatively low intensity (17% of the total intensity). *Marinobacter* in turn also strongly affected bacterial community composition as there were pronounced shifts in band identity and intensity between the treatments with inocula alone and with inocula + *Marinobacter*.

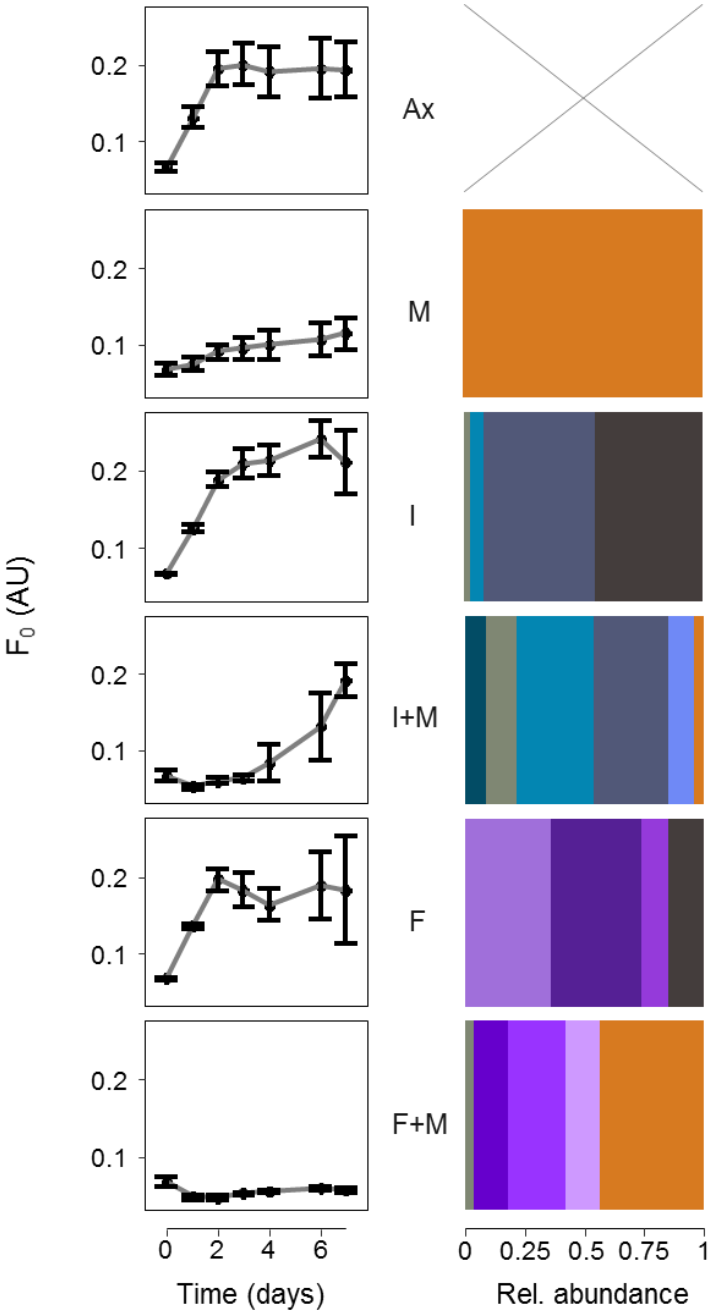

Figure 3: Natural bacterial inocula modulate the growth-inhibiting effect of a Marinobacter strain on the diatom *Cylindrotheca closterium.* Diatom biomass accumulation and bacterial community composition (on day 7) are shown for the different treatments (Ax= axenic, M= *Marinobacter* strain SrVMe alone, I= intertidal bacterial inoculum alone, F= forest soil bacterial inoculum alone, I+M= *Marinobacter* and intertidal inoculum, F+M *Marinobacter* and forest soil inoculum). Diatom biomass (left, measured as F_0 using PAM fluorometry) is expressed in arbitrary units (AU) of fluorescence. The stacked bar plots (right) show the results of the DGGE analyses of the bacterial communities. The width of the bars is proportional to the relative intensity of the bands in the DGGE gels. Each colour represents a unique DGGE band (orange = SrVMe, shades of blue = bands unique to intertidal inoculum, shades of purple = bands unique to the forest inoculum, grey = bands present in both inocula).

Discussion

The isolation of bacteria from various benthic diatom cultures resulted in a strain collection with representatives from most groups that are commonly found in diatom-bacteria consortia (Amin et al., 2012), such as the Gram-negative Alphaproteobacteria, Gammaproteobacteria and Flavobacteriia, and the Gram-positive Actinobacteria. A clear dominance of Alpha- and Gammaproteobacteria was observed, which is in agreement with other studies on the composition of bacterial assemblages in diatom cultures (Behringer et al., 2018; Kaczmarska et al., 2005; Sapp et al., 2007). We did not obtain any isolates of several other groups which have previously been reported from diatom cultures, such as the Betaproteobacteria (Baker and Kemp, 2014; Bruckner et al., 2008). The fact that only a few bacterial genera were obtained from multiple diatom cultures may suggest that the bacterial communities associated with the original diatom cultures were distinct from one another.

The growth responses of the diatoms to the bacteria ranged from strongly negative over neutral to positive. However, most of the bacteria elicited a negative growth response. This might, in part, be due to the fact that we used a complete (including excess vitamins and bio-available iron) and nutrient-rich medium for the incubations, which may mask potentially beneficial effects of symbiotic bacteria (Croft et al., 2005; Soria-Dengg et al., 2001). However, a screening of bacterial effects on the growth of the green microalga *Dunaliella*, in which no vitamins were included in the media, showed a similar prevalence of negative interactions (Le Chevanton et al., 2013). Furthermore, less nutrients would not necessarily promote symbiotic interactions as they might simultaneously enhance competition between bacteria and host (Grossart, 1999). Alternatively, the relatively rich medium on which bacteria were isolated might have resulted in an enrichment for more opportunistic bacteria.

Only bacteria that were not isolated from *C. closterium* strain WS_{3_7} could significantly reduce the growth rate of this diatom strain. In general, bacteria isolated from diatom species other than *C. closterium* had a higher chance to be growth inhibiting. These observations are suggestive of coadaptation, which is expected to result in a higher host fitness when naturally co-occurring partners are combined than in combinations with foreign partners (Sison-Mangus et al. 2014). Such coadaptations could include niche differentiation, whereby coadapted partners use different substrates (Tuchman

et al., 2006) to reduce competition, or the release of signalling compounds by the host which suppresses the production of algaecides by the bacteria (Demuez et al., 2015).

Several hypotheses have been proposed to explain how coadaptation between microalgae and their microbiome, often in the form of interdependencies between partners (e.g. Amin et al. 2015), originates (Kazamia et al., 2016; Morris et al., 2012). However, to date it remains unclear whether such interdependencies result from coevolution or whether they can also arise from more transient interactions (Cooper et al., 2019; Kazamia et al., 2016). If such coadaptations would result from a shared evolutionary history between a host and its associated bacteria (i.e. coevolution *sensu stricto* as in Herrera (1985)), we would expect the phylogenetic position of the diatom hosts to reflect their growth response to bacteria. However, this was not observed. The lack of a phylogenetic signal in the growth response of the diatoms could have several underlying causes. The interactions could be transient, decoupled from the evolutionary dynamics of the bacteria and diatoms. The ease with which diatoms could be cultivated axenically in this study, but also in other studies (e.g. Behringer et al. 2018), supports this claim. Transience of the interactions would imply that suitable (coadapted) partners need to recognize one another and be able to establish interactions, which might require specific molecular mechanisms, including as chemotaxis (Seymour et al., 2017; Sonnenschein et al., 2012). Alternatively, the adaptation of bacteria to their algal host could be occurring too fast to result in a deeper phylogenetic signal (Revell et al., 2008). In other systems, adaptation of bacteria to their host happened rapidly, within months (Zdziarski et al., 2010; Zhou et al., 2017). In our study, the bacteria were isolated from unialgal diatom cultures which had already been maintained in the lab for up to several months, during which coadaptation could therefore have occurred. Further research, in which the effect of co-cultured bacteria on the growth of their algal host is evaluated over time, could show if this has indeed been the case.

The effect of bacteria on diatom growth not only depended on the relationship between the host and its associated bacteria (i.e. own vs foreign), but also strongly differed between bacterial groups, with some groups tending to contain more growth-inhibiting representatives than others. Strains belonging to the *Flavobacteriia* and *Gammaproteobacteria*, two common phycosphere- and biofilm-associated bacterial groups (Seymour et al., 2017)(Pollet et al., 2018), comprised several strong growth

inhibiters. One of the strongest growth inhibiters was *Croceibacter* strain SrVMc (Flavobacteriia). Several growth-inhibiting *Croceibacter* strains have been reported before (Van Tol et al., 2017) and strain SrVMc was also shown to reduce the mating success of its original diatom host (*Seminavis robusta*), but not its growth (Cirri et al., 2018). Two out of the four *Marinobacter* strains also strongly reduced growth. *Marinobacter* representatives have been reported to range from commensal (Amin et al., 2015) to growth-promoting (Amin et al., 2009; Gärdes et al., 2012) and growth-inhibiting (Wang et al., 2014). The latter study reported that growth inhibition occurred by impeding cell division in the diatoms, similar to what we observed for *Marinobacter* strain SrVMe. This growth-inhibitory effect might benefit the strain by inducing physiological changes in the host which enhance the release of metabolites required by the bacterium (Van Tol et al., 2017). We observed marked variability between closely related *Marinobacter* strains in their effect on diatom growth. Sher et al. (2011) found that while in general the effects of heterotrophic bacteria on the growth of *Prochlorococcus* were phylogenetically cohesive (i.e. similar between closely-related bacterial strains), differences in algal growth responses existed between different *Marinobacter* strains. This variability could be related to the genes involved being located on plasmids (Smillie et al., 2010) or to the abundance of mobile genetic elements in their genome (Gärdes et al., 2010; Singer et al., 2011). Similar genetic mechanisms may also be responsible for the loss of the growth-inhibiting capacity observed in *Croceibacter* strain SrVMc, and can generally affect the dynamics of diatom-bacteria interactions by e.g. promoting lateral transfer of genes involved in the host-bacteria interaction.

None of the nine *Rhodobacteraceae* (*Alphaproteobacteria*) strains, a group suggested to be strongly adapted to algae (Sonnenschein et al., 2018), had pronounced effects on the growth of *C. closterium* WS_{3_7}. This family has previously been recognised as the most abundant family associated with diatoms (Geng and Belas, 2010) and was present in at least four out of the six *C. closterium* cultures. Several members of this family have been reported as algal growth inducers (Amin et al., 2015) or inhibitors (Barak-gavish et al., 2018; Sonnenschein et al., 2018).

The interactions reported above were all made in a simplified experimental context, which is very different from the natural situation. *In situ*, diatoms have many different bacteria attached to them (Baker and Kemp, 2014), and interactions between these bacteria and with their hosts can modulate individual interactions (Dittmann et al.,

2018). We showed that the strong inhibiting effect of *Marinobacter* strain SrVMe (isolated from the diatom *Seminavis robusta*) on a *C. closterium* strain rapidly dissipated in the presence of other intertidal marine bacteria (obtained from the same locality as the *C. closterium* strain) but was enhanced in the presence of a soil bacterial inoculum. A potential explanation is that the *Marinobacter* strain was outcompeted by the tidal but not the soil inoculum. However, it could also be that the diatom host actively recruited specific bacteria to counteract the *Marinobacter* (Ford and King, 2016), a hypothesis which is supported by the compositional shift in the *C. closterium* microbiome in the presence of the *Marinobacter* strain. Such a mechanism would reinforce the specificity between diatoms and their microbiome, favouring the establishment of beneficial bacteria, as observed in this study.

Acknowledgements

W. Stock was funded by the Research Foundation – Flanders (FWO). S. D'Hondt assisted us with the molecular work. This research was supported under the BIO-Tide project, funded through the 2015-2016 BiodivERsA COFUND call for research proposals, with the national funders BelSPO, FWO, ANR and SNSF. The research leading to results presented in this publication was carried out with infrastructure funded by EMBRC Belgium - FWO project GOH3817N. Frederik De Boever is acknowledged for his experimental work and his usefull feedback on the manuscript. E. De Brandt and L. Lebbe assisted in the isolation of the bacteria and the maintenance of these isolates afterwards.

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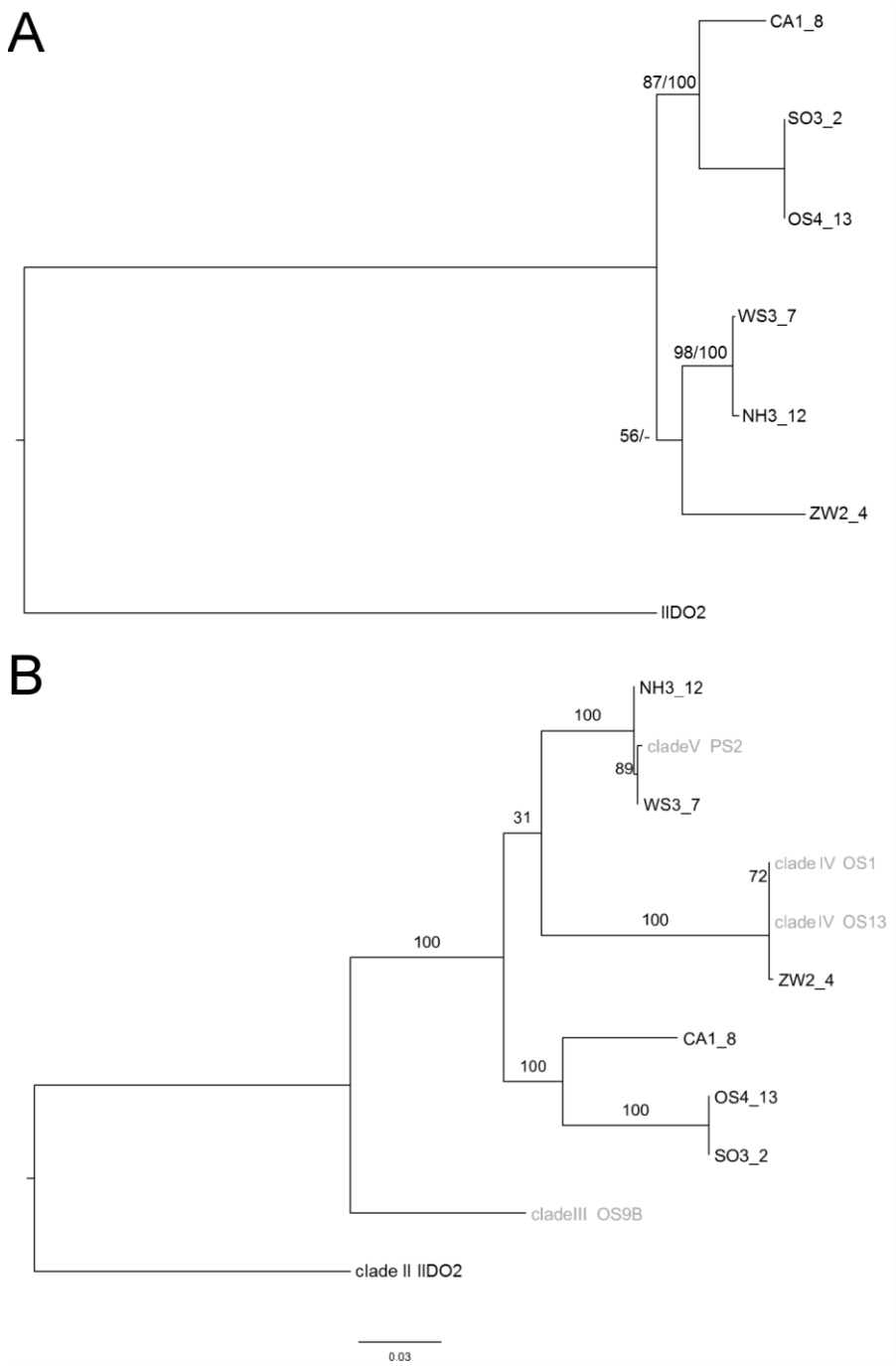
Supplementary material

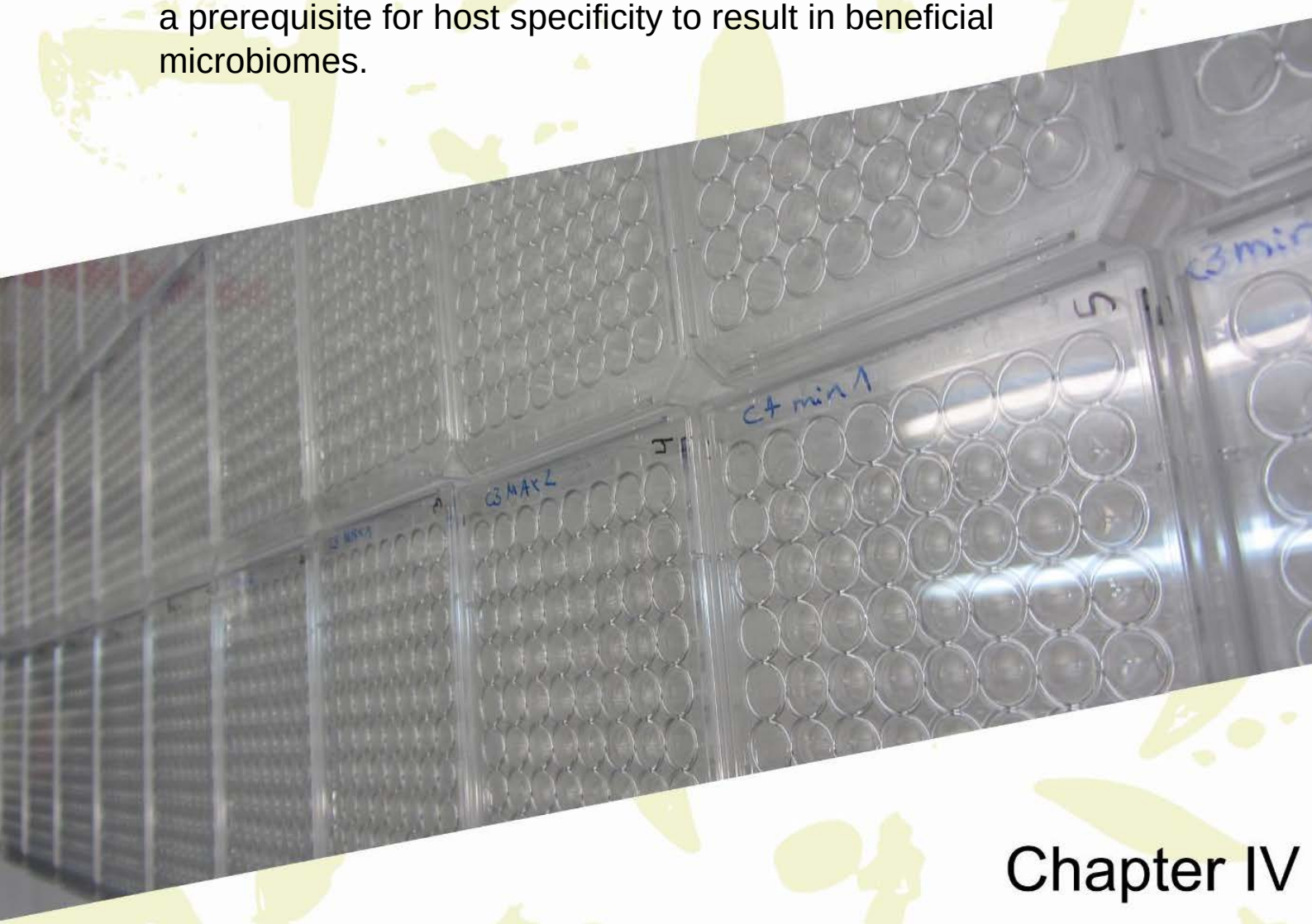
Supplementary table 1:Origin of the *Cylindrotheca closterium* and *C. fusiformis strains* used in this study

Id	Species	Location	Latitude	Longitude
SO_{3_2}	*C. closterium*	Bay of the Somme (FR)	N 50° 12’ 54,42”	E 1° 34’ 15,6”
CA_{1_8}		Bay of the Canche (FR)	N 50° 32’ 14,46”	E 1° 35’ 36,06”
ZW_{2_4}		The Zwin plain (BE)	N 51° 21’ 55,8”	E 3° 21’ 51,42”
WS_{3_7}		Wester Scheldt (NL)	N 51° 21’ 1,92”	E 3° 43’ 34,44”
OS_{4_13}		Eastern Scheldt (NL)	N 51° 31’ 48,78”	E 3° 56’ 40,14”
NH_{3_12}		North Holland (NL)	N 52° 53’ 46,68”	E 4° 54’ 31,38”
IIDO2	*C. fusiformis*	Wester Scheldt (NL)	N 51° 19'	E 4° 16'



Supplementary figure 1: consensus maximum likelihood (ML) trees with the *Cylindrotheca* strains used in this study. IIDO2, a *Cylindrotheca fusiformis* strain, was set as outgroup in both phylogenies. (a) Only strains used in the experiments of this study, with ML-based bootstraps and (for corresponding nodes only) Bayesian Inference (BI) probabilities for each node. (b) Tree including several other strains from Vanormelingen et al. (2013). These are indicated in grey. ML based bootstrap displayed for each node.

Chapter IV: Coadaptation between diatoms and their bacteria is a prerequisite for host specificity to result in beneficial microbiomes.



Chapter IV

Coadaptation between diatoms and their bacteria is a prerequisite for host specificity to result in beneficial microbiomes

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Abstract

Diatoms, like many organisms, live in close association with bacteria. To test if specifically associated bacteria contribute to an increased fitness of the host, two benthic diatom species, represented by different strains, were exposed to microbiomes initiated from different natural inocula and assembled during contact with different hosts. The initial assembly process was accompanied by a reduction in competition between the primary host and the bacteria, reflected by a gradual growth recovery of the host following initial contact, and the subsequent development of species-specific microbiomes. When secondary hosts were challenged by the microbiota from the primary hosts, their impact depended on the relatedness of the current and initial host, but also on the origin of the bacterial community. Sympatric bacteria tended to decrease competition with their secondary host if it was phylogenetically more similar to their primary host. Allopatric bacteria, on the other hand, did not show this pattern, even though these bacteria were also recruited into host-specific microbiomes. These results suggest that diatoms have species-specific bacterial communities but co-adaptation between host and bacteria is required for the specificity to result in a reduced competition with its host. These findings contribute to an improved understanding of the microbiome assembly process and shed a new light on the host specificity observed in microbiomes.

Introduction

Throughout their evolution, diatoms interacted closely with bacteria, as evidenced by the frequent lateral gene transfer from bacteria to diatoms (Armbrust et al. 2004; Bowler et al. 2008) and many recent studies demonstrating intimate relationships between them (e.g. Amin et al. 2015; Durham et al. 2017). Diatoms benefit from the nutrients recycled by bacteria (Buchan et al. 2014), but also rely on bacteria to alleviate oxidative stress (Morris et al. 2012; Hünken et al. 2008) and for the acquisition of iron (Amin et al. 2009) and vitamins (Helliwell et al. 2013). Simultaneously, diatoms provide bacteria with organic carbon (Mühlenbruch et al. 2018; Taylor & Cunliffe 2017). However, due to a considerable overlap in ecological niches, diatoms and bacteria also compete for resources (Diner et al. 2016; Risgaard-petersen 2004). It is becoming increasingly clear that different diatom species tend to harbour different bacteria (Behringer et al. 2018; Grossart et al. 2005; Sison-Mangus et al. 2014), which might reflect the evolutionary outcomes of the costs and benefits resulting from specific diatom-bacteria interactions.

In addition to being associated with different bacteria, diatoms tend to have very specific interactions with bacteria. Paul & Pohnert (2011) showed how one bacterial strain was algicidal to one diatom species but not to another. Conversely, Amin et al. (2015) discovered that a *Sulfitobacter* species could strongly enhance the growth of a *Pseudo-nitzschia multiseries*, but not the growth of several other diatoms. Moreover, two tested *Phaeobacter* strains, closely-related to *Sulfitobacter*, did not promote growth of the aforementioned *Pseudo-nitzschia* strain. In return, diatoms can alter their behaviour and carbon release in a highly specific manner as a response to the bacteria present (Gärdes et al. 2011). A bacterial transplant experiment showed that bacteria were mutualistic to their native diatom host but commensal or even harmful to a foreign host (Sison-Mangus et al. 2014). In response to the foreign bacteria, the diatoms increased toxin production. Based on these findings, we theorize that the specificity in the interactions between diatoms and bacteria results from adaptations that both partners have developed to select and interact with appropriate partners.

It is generally assumed that intimate, non-parasitic associations between hosts and microorganisms are the result of eco-evolutionary selective and adaptive processes during which relations between interacting organisms shift towards less competition and more cooperation (Good et al. 2018). Strong competition between

microorganisms can be rapidly attenuated by competitive exclusion (Rivett et al. 2016). Furthermore, since interacting organisms are under selection to minimize any kind of conflict between them (Costello et al. 2012), even weak competitive interactions are expected to dissipate over time through niche differentiation (Good et al. 2018). In addition, extended coexistence between organisms is expected to favour the development of mutualisms (Zhang 2003) and dependencies (Morris et al. 2012), thereby enhancing the potential for cooperation. Examples of niche differentiation (Tuchman et al. 2006) and extensive metabolite exchange between diatoms and bacteria (Amin et al. 2015; Helliwell et al 2013) are accumulating, illustrating how eco-evolutionary dynamics may foster cooperation over competition between coexisting diatoms and their bacteria, thus reflecting extensive coadaptation. In the present study, we hypothesize that community assembly in diatom-associated bacterial consortia will be constrained by the tendency for commensal or mutualistic relationships to become dominant. Community assembly will thus mainly be driven by selection for bacteria that minimize competition with the host and maximize mutually beneficial interactions. Such bacterial communities will mainly be composed of bacteria that have a history of coadaptation with their host, which has resulted in the development of specific commensal and mutualistic interactions. We thus predict that a diatom will only be able to assemble a beneficial, specific microbiome from a bacterial community if that community contains bacteria that are sufficiently adapted to interact with the diatom. To experimentally test our hypotheses, we set up experiments in which we inoculated axenic diatom strains, belonging to two marine benthic species, with different sympatric (i.e. potentially containing bacteria adapted to their host) or allopatric (i.e. not containing bacteria adapted to their host) natural bacterial communities (Figure 1).

We first performed a selection assay in which the natural bacterial inocula were cocultured with axenic diatom strains (the primary hosts) and bacterial community assembly was examined during six consecutive growth cycles. In a second assay (fitness assay), we then cocultured secondary diatom hosts (with different degrees of phylogenetic relatedness) with the bacterial communities isolated from the primary hosts at the end of the selection assay. As most studies have thus far indicated that different diatoms have different microbiomes (e.g. Behringer et al. 2018), we expected the sympatric inocula to develop into diatom species-specific microbiomes during the

selection assay. Moreover, we expected these assembled microbiomes to be less negative or more beneficial to the secondary host, if this host was more closely related to the first host. For microbiomes initiated from allopatric inocula, we did not expect the resulting microbiome to be species-specific nor to be more beneficial if primary and secondary host were the same species due to the absence of adapted bacteria.

As the strength of the interactions between bacteria and their algal host may be dependent on the environment (Grossart 1999), we exposed the diatoms to the bacterial inocula in two different resource environments: one with a low supply of inorganic nutrients and one with a high supply of inorganic nutrients and additional organic carbon, vitamins and bioavailable iron. We expected the more stressful, less nutrient rich conditions to amplify the outcome of diatom-bacteria interactions: beneficial bacteria were expected to reduce nutrient stress on the diatoms as was previously observed in plants (Lau & Lennon 2012), whilst competitive interactions might be reinforced by the nutrient limitation (Scott et al. 2008).

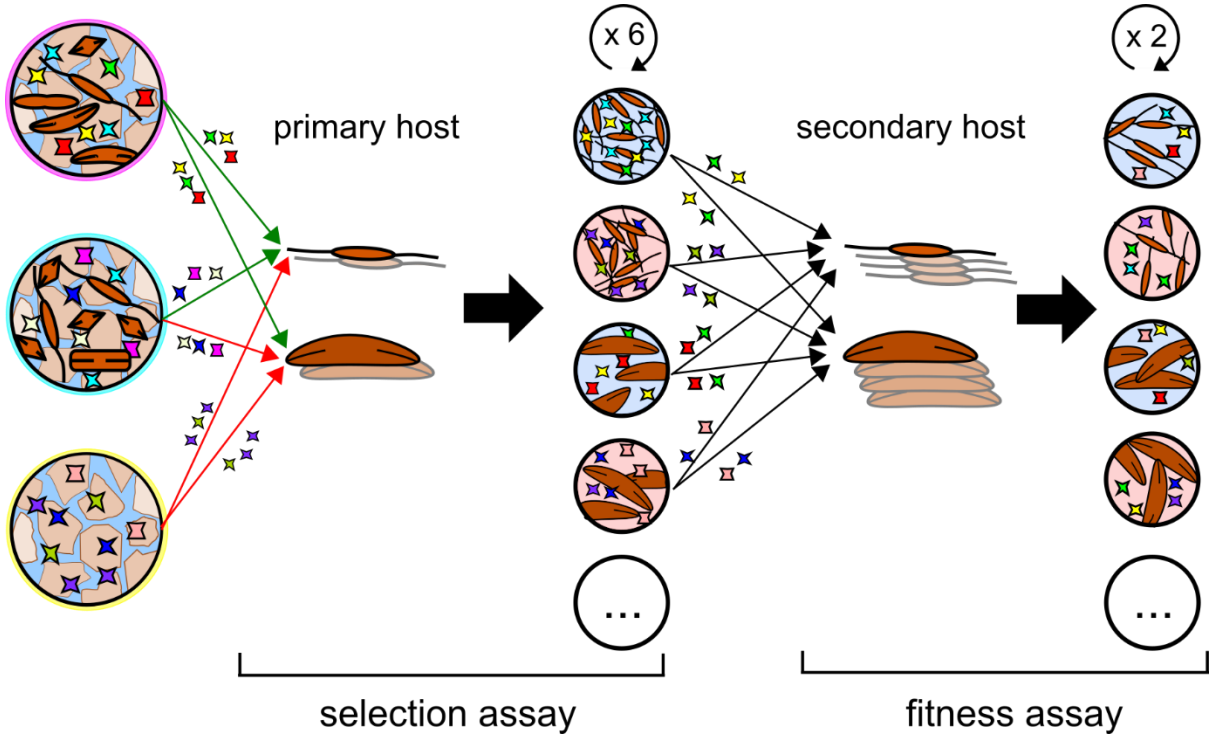

Figure 1: Schematic representation of the experiment. Axenic diatoms were inoculated with bacteria, coming from three different locations where these diatoms naturally occurred (sympatric, green arrows) or not (allopatric, red arrows). These diatoms (the primary hosts) were cocultured for six consecutive growth cycles (the selection assay) in either a high (red) or a low (blue) resource medium. After which the bacterial communities were removed from their primary host and given to a secondary host. The growth of the secondary hosts was monitored for another two consecutive growth cycles (the fitness assay).

Changes in the bacterial community composition were assessed using high-throughput 16S rDNA amplicon sequencing. As we could not distinguish the bacteria intrinsically associated with the diatoms from those less dependent on their host, we considered the bacterial community as a whole to be diatom-associated. The effects of the bacterial communities on the fitness of their primary and secondary host was monitored through Imaging-PAM fluorometry, a robust method to measure and compare diatom growth (appendix I). We expected an increase of diatom growth to reflect a higher fitness of the host due to mutualistic effects of bacteria on their host, such as increased bioavailability of limiting nutrients (Seymour et al. 2017) and a reduced diatom growth to reflect a fitness decrease of the host due to competition with its bacteria. Competition between host and bacteria could be direct whereby bacteria actively damage the host, as is for instance the case with algicidal compounds (Meyer et al 2017) or indirect by competing for resources (Ghoul & Mitri 2016).

Material and methods

Diatoms

Two marine benthic species were used in this study, namely *Seminavis robusta* and *Cylindrotheca closterium*. For each species, four different strains were obtained from the BCCM/DCG culture collection (Table 1). The four *S. robusta* strains all belonged to the same mating group (De Decker et al. 2018).

Diatoms were, prior to the experiment, grown in autoclaved natural seawater supplemented with Guillard's Marine Water Enrichment Solution (Sigma Aldrich). They were maintained at 18°C in 12:12h light-dark cycles using cool fluorescent white light (16-19 µmol photons/s/m²). To rear the diatoms axenically, they were repeatedly treated with antibiotics. Antibiotic treatments coincided with the medium refreshments which occurred every three days. The treatments consisted of adding 500 µg /ml penicillin, 500 µg /ml ampicillin, 100 µg/ml streptomycin and 50µg/ml gentamycin (Sigma Aldrich) and were repeated at least three times. DAPI staining (Shishlyannikov et al., 2011) and plating on Difco Marine agar (BD) was used to confirm axenicity of the diatom cultures.

Table 1: algal strains used in this study

S. robusta			*C. closterium*		
Identifier	BCCM/DCG ref.	Used in the selection assay	Identifier	BCCM/DCG ref.	Used in the selection assay
S1	VM3-4 - DCG 0514		C1	WS3_7 - DCG 0623	
S2	VM3-16 - DCG 0550	X	C2	NH3_12 - DCG 0621	
S3	VM3-15 - DCG 0545	X	C3	ZW2_12	X
S4	85A - DCG 0105		C4	NH3_1	X

Media

A high and a low resource marine medium were used in the experiment. Both were based on a modified version of the diatom artificial medium (DAM). DAM was prepared as described (Gagneux-Moreaux et al. 2007) apart from the vitamin solution and EDTA which were both omitted. This solution was, after autoclaving, diluted with 10% artificial seawater (Tropic Marine) to be used as the low resource medium. The high resource medium was prepared by adding 5ml/l of Guillard's Marine Water Enrichment Solution, which contains additional nutrients, bioavailable trace elements, vitamins B1, H and B12, and 0.05g/l yeast extract to the low resource medium.

Bacterial inocula

Intertidal sediment was collected from a brackish mudflat along the Westerscheldt estuary (WS, N 51°21' 1,9" E 3°43' 34.4") during low tide. A subtidal marine biofilm (growing together with macroalgae) was scraped of a pontoon in the Veerse Meer (VM, N51°31'32,6" E3°47'54.8"). The biofilm was collected together with the surrounding water. Plant soil (PS) was collected from a disturbed forested area in Ghent, Belgium (N51°01'27" E3°42'42") (Chapter III). The bacterial communities, used to inoculate the diatom cultures, were prepared from these three environmental samples. For the WS and PS samples, a 20g aliquot was taken and supplemented with low resource marine medium to a final volume of 140ml. Thirty-five ml of the VM biofilm-seawater mixture was supplemented with low resource medium to the same volume. Bacteria were detached from the sediment particles and biofilm by 30s of vortexing alternated with 1 min of shaking which was repeated twice. After centrifuging for 10min at 250 RCF, the supernatant was filtered twice over a sterilised 2.5µm paper filter (Whatman) to remove particles and larger eukaryotic organisms. The three bacterial suspensions (filtrate) were treated with final concentration of 50 µg/ml neutral red (Sigma-Aldrich) overnight (18°C, dark) to kill any remaining eukaryotes (Shimeta & Cook 2011). To remove neutral red, the solution was filtered over a sterilized 0.1µm membrane filter and washed five times with low resource medium. The bacteria on the filter were resuspended in low resource medium and diluted to the same bacterial densities based on DAPI counts (Shishlyannikov et al., 2011). A mixed inoculum (MIX) was composed by mixing equal volumes of each three suspensions. These four (MIX, WS, PS and VM) bacterial communities were used source communities to inoculate the axenic diatoms in the selection assay.

C. closterium is common in both the WS and VM locality (appendix II; Vanelslander et al. 2009), while *S. robusta* is common in the VM locality (De Decker et al. 2018). Both species are absent from the PS locality.

Selection assay

For the selection assay, two of the four available strains of each diatom species (S2 & S3 and C3 & C4; the primary hosts) were inoculated with the bacterial source communities (MIX, WS, PS and VM). The four axenic cultures were growing exponentially prior to the experiment. Their medium was refreshed by pipetting off the old medium after centrifuging the culture (900RCF, 12 min) and adding fresh low

resource medium. This was repeated once to ensure complete medium refreshment. Cultures were diluted to a final density of approximately 20 000 cells/ml. The assay was setup in transparent 24 well plates (Greiner CELLSTAR, Sigma-Aldrich). All four diatom strains (S2 & S3 and C3 & C4) were combined with each of the four different bacterial inocula (PS, VM, WS and MIX) by adding one ml of diatom culture and 0.5ml of bacterial source community. The final volume of all wells was set to 2 ml by adding medium (enriched or not) to obtain the composition of the low or high resource medium. All combinations (2 strains × 2 diatoms × 4 bacterial inocula) were made in triplicate for both low and high resource medium, resulting in 96 diatom cultures. Additional controls were setup in which only the bacteria, the axenic diatom strains or neither bacteria nor diatoms (blanks) were added. All treatments were randomised over the well plates.

Every week, for five consecutive weeks (growth cycles), all cultures were diluted and transferred to new well plates. Therefore cultures were first re-suspended by scraping, followed by pipetting up and down. The amount of culture transferred to the new well was adjusted to again obtain starting densities of 20 000 diatom cells in the new wells. If cultures were too dilute to obtain sufficient algal biomass in the new well, half the culture (1ml) was transferred to the new well.

Of the remaining re-suspended culture, 750µl was transferred to a sterile Eppendorf tube and pelleted by centrifugation (5478 RCF, 10min). Pellets were stored at -80°C for 16S rDNA analyses. In addition to a subsample from the bacterial source communities used to inoculate wells, samples from the first, third and sixth week were selected for DNA analysis. The DNA was extracted, amplified and sequenced as described earlier (Chapter II). Ten µl of all axenic cultures and blanks was plated on marine agar to check for bacterial contamination. Agar plates were incubated at 18°C and checked after six days.

After six weeks, one replicate bacterial community of every combination (medium × diatom strain × inoculum) (Table 2) was used in the fitness assay (see below).

Table 2: the different treatments of the selection assay which resulted in the bacterial communities to be used in the fitness assay.

Media	Diatom Strain	Bacterial inocula
Low	*S. robusta* S2	WS
High	*S. robusta* S3	VM
	C. closterium C3	PS
	C. closterium C4	MIX
	No diatom present	

Fitness assay

After six weeks of semi-continuous batch culturing, bacterial communities from the selection assay were harvested. They were diluted to the same algal density (20 000 algal cells/ml) followed by repeated vortexing to detach bacteria from the diatoms. The primary hosts themselves were removed through filtration (Acrodisc 5µm Supor Membrane Syringe Filter, PALL).

For the fitness assay, ninety-six-well plates (Greiner CELLSTAR, Sigma-Aldrich) were inoculated with axenic diatom strains (now all four *C. closterium* and all four *S. robusta* strains; the secondary hosts) to the same final algal densities per area as before. A fully-crossed design was setup in which all the diatom strains were combined with the bacterial communities from the selection assay in both low and high resource medium. Axenic controls for all diatoms were also included. All treatments were setup in triplicate. After one week, all cultures were refreshed as was done for the selection assay (two growth cycles).

Monitoring the cocultures

Diatom growth during every growth cycle of the selection assay (growth of the primary host) and the fitness assay (growth of the secondary host) was monitored through pulse-amplitude modulated (PAM) fluorometry (Appendix I). Daily measurements of the minimal fluorescence (F_0; PAM settings: measuring intensity 5, gain 2) were used to estimate the diatom biomass per well. This was done for the six cycles of the selection assay and the subsequent two cycles of the fitness assay. All cultures were checked microscopically for potential (cross)contaminations on a regular basis. Contaminated cultures were discarded.

Data analyses

For every growth cycle in both the selection (6 growth cycles) and the fitness assay (2 growth cycles), the maximal growth rate of the diatoms was calculated for all wells. Growth rate was calculated as the linear increase of the log2-tranformed algal biomass for every three consecutive days (moving window). Only the highest observed growth rate (maximal growth rate) was retained for further analyses. The influence of the host type (*S. robusta*, *C. closterium* or no diatom present), inoculum and medium type was evaluated for each cycle of the selection assay in a random forest model (randomForest 4.6). This machine learning technique forms a non-parametric alternative to regression based statistics (Breiman 2001), whereby the increase in mean squared error (MSE) of each variable when randomly permuted by another variable was used as a proxy for the importance of each variable. For the fitness assay, the random forest models were constructed in a similar fashion, but in addition to the three factors used in the selection assay, the influence of the primary host (=host in the selection assay), its relation to the secondary (=host in the fitness assay) host (i.e. same strain or species), the previous medium (=medium in the selection assay) and its relation to the fitness assay medium (same or different), were also included.

The significance of these terms and the interactions between them were tested for each species separately in a mixed linear model with the strain as random effect (lmer - lme4 1.1).

The 16S rDNA sequence data was processed as described earlier (Chapter II), which the exception of the dereplication of the sequences which was done in vsearch (Rognes et al. 2016) instead of usearch8 (Edgar 2010). The operational taxonomic units (OTUs) were classified in MOTHUR (Version 1.32.1; Schloss et al. 2009) using the May 2013 GreenGenes training set (DeSantis et al. 2006; McDonald et al. 2012). The abundance of bacteria relative to the algal abundance was calculated as the number of reads assigned to bacteria divided by the number of reads assigned to algal chloroplast in a sample. For further analyses, all reads assigned to chloroplasts or mitochondria were discarded. Samples with less than 2000 residual reads were discarded and the remaining samples were rarefied to 2379 reads. The data was visualised through two-dimensional Nonmetric Multidimensional Scaling (NMDS) using the Bray-Curtis distances between samples. Diversity within samples (alpha-diversity) and between samples (beta-diversity) was calculated as the Shannon

diversity and the Bray-Curtis distances respectively. Mixed effects models (lmer - lme4 1.1), with the inoculum, medium type and diatom species as random effects, were used to test for linear changes in both indices over the growth cycles in the selection assay. The influence of the medium, inoculum, diatom (*S. robusta*, *C. closterium* or no diatom present) and growth cycle (as factor) on the bacterial communities was tested through a PERMANOVA (9999 permutations; Adonis - Vegan 2.4), considering all possible interactions between these factors. A variation partitioning was used to resolve the explanatory power of each these variables (varpart – Vegan 2.4). For both the PERMANOVA and variation partitioning, the wells inoculated with the MIX bacterial community were not considered to ensure independence of bacterial source communities. The number of OTUs shared between bacterial communities during the selection assay was compared between the different inocula and diatom species over the growth cycles. OTUs were considered shared between communities if they occurred in at least 3 independent samples of each group.

Results

Bacterial inocula

The bacterial inocula differed substantially from one another (Figure 2; Supplementary Figure 1). The PS community was dominated by Clostridiales. The MIX and WS communities lacked Clostridiales but were dominated by Rhodobacterales. The VM community on the other hand was dominated by Actinomycetales.

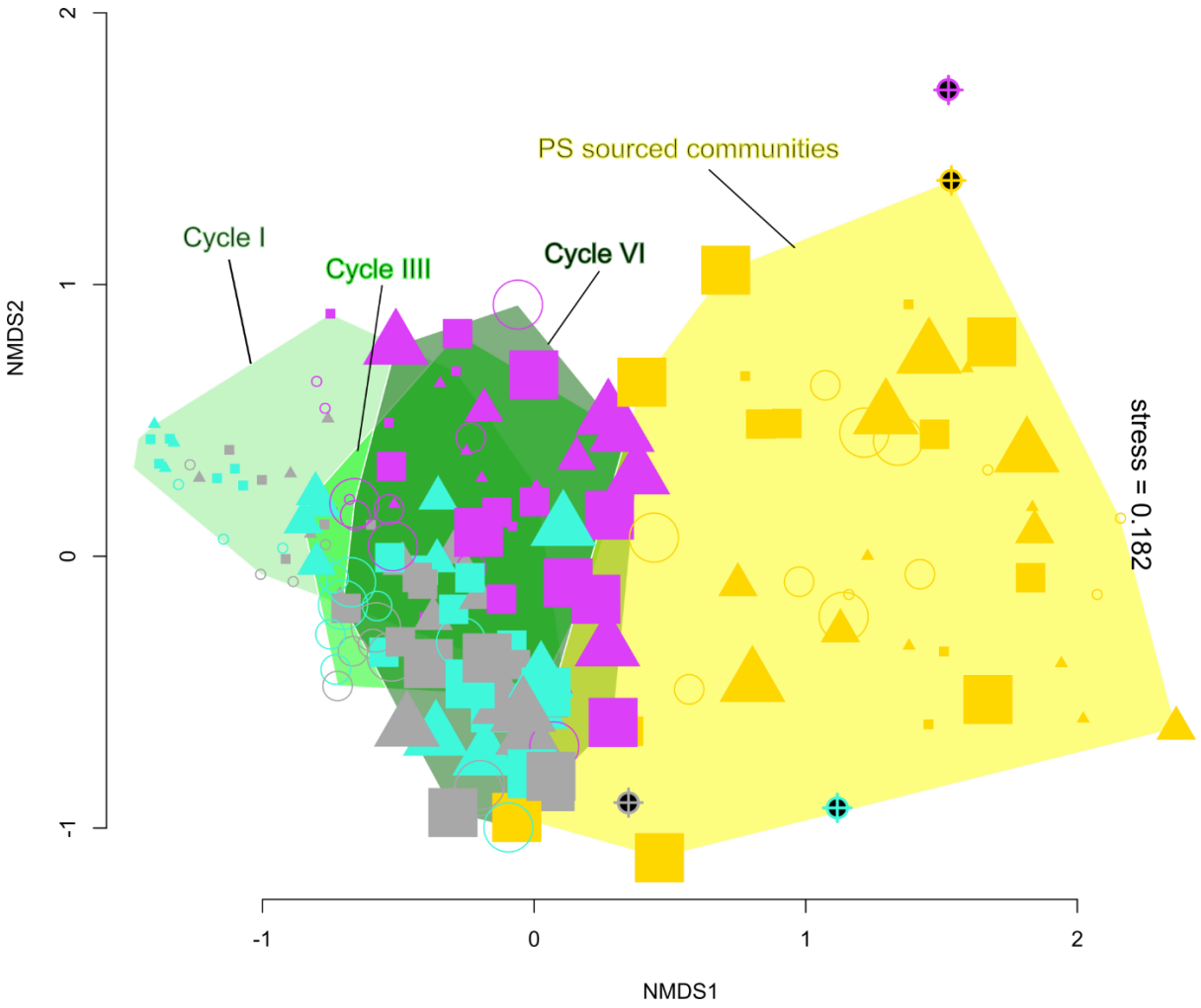

Figure 2: Dynamics at the order level between microbiomes. Non-metric multi-dimensional scaling (NMDS, 2D) displaying the Bray-Curtis dissimilarity based differences between the microbiomes during the consecutive growth cycles of the selection assay. The colours of the symbols represent the different bacterial source communities (cyan for WS, purple for VM, yellow for PS, grey for mixture of the three sources) whilst the shape of the symbols signifies the different hosts (circle: no diatom; square *C. closterium*; triangles *S. robusta*). Size of the symbols increases with the cycle, i.e. the smallest symbols match the microbiomes after the first cycles of the selection assay, whilst the largest match those after the sixth cycle. The green polygons encompass all microbiomes from the VM, WS and MIX sources per growth cycle as indicated in the figure. All PS microbiomes are enclosed by the yellow polygon. The black shaded crosshair indicate the position of the bacterial inocula. The two-dimensional stress is shown on the right.

Selection assay

After the first growth cycle of the selection assay, *Arcobacter* OTUs (Campylobacterales) dominated the bacterial communities derived from the MIX and WS inocula (Supplementary Figure 1). *Arcobacter* was also the only genus in common

between all inocula after both the first and the third cycle (Supplementary Figure 2). The abundance of this genus generally decreased over time, but it remained abundant in the 'no diatom' treatments with high resource medium until the end of the selection assay. From the third cycle onwards, the proportion of Rhodobacterales increased. The most common OTUs from this order were assigned to *Phaeobacter gallaeciensis* and *Celeribacter baekdonensis*. Note that the *Celeribacter* was almost absent in the VM inoculated communities whilst the presence of *Phaeobacter* was neglectable in the PS inoculated communities. Other abundant orders during the selection assay included Flavobacteriales, which were particularly abundant in the wells inoculated with the VM inoculant. This order persistently had a higher abundance in the *C. closterium* than the *S. robusta* cultures. Whilst the MIX, VM and WS communities displayed relatively similar dynamics, at least when considered at a lower taxonomic resolution (Figure 2), the wells inoculated with the PS community had a different composition and dynamic. They were initially dominated by Pseudomonadales, mainly *Pseudomonas* spp., and from the third cycle onwards by Rhizobiales.

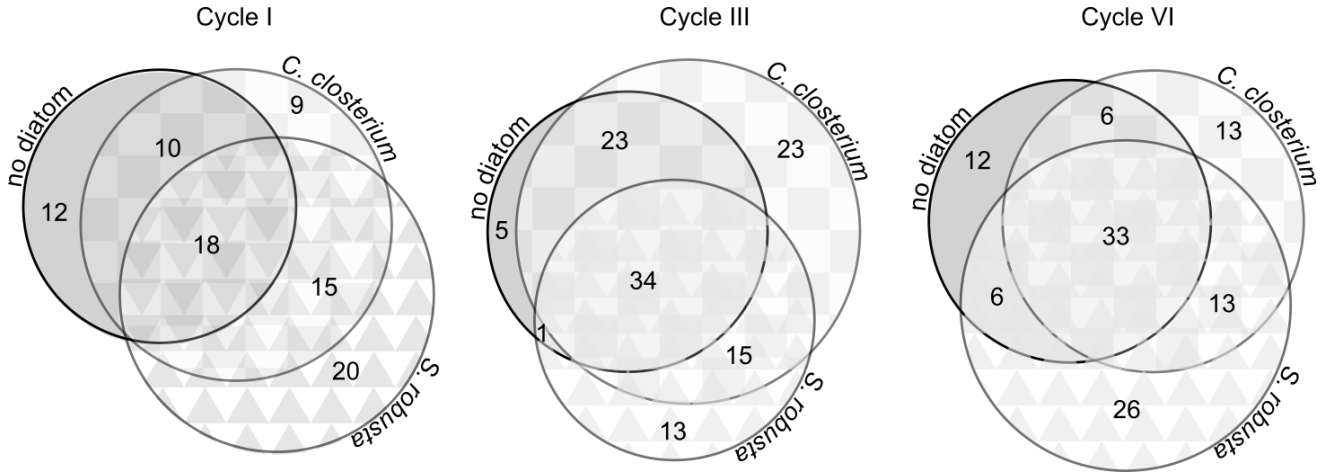

Figure 3: Most bacterial OTUs are shared between both diatoms and the 'no diatom' control. The number of OTUs shared (occurring in at least three samples of the group) between the diatom species and the control during the first, third and sixth growth cycle of the selection assay.

Many OTUs were shared between the communities derived from the different inocula or between the host treatments. The control (no diatom) shared a relatively higher portion of OTUs with *C. closterium* and *S. robusta* (Figure 3). The OTUs shared or unique to each of these groups were assigned to different bacterial orders, without an obvious taxonomic bias between these groups (data not shown). The MIX inoculated

bacterial communities generally resembled the WS inoculated communities and generally shared most OTUs with the WS inoculated communities. Apart from the OTUs in common between all bacterial communities derived from the different inocula, the MIX inoculated communities shared no OTUs with the PS community (Supplementary Figure 2).

During the growth cycles of the selection assay, the relative abundance of the bacteria in relation to the diatoms, calculated as the ratio of bacterial reads to reads assigned to the algal chloroplasts, decreased substantially: the median ratio decreased 200-fold between the first and the last cycle (Supplementary figure 3).

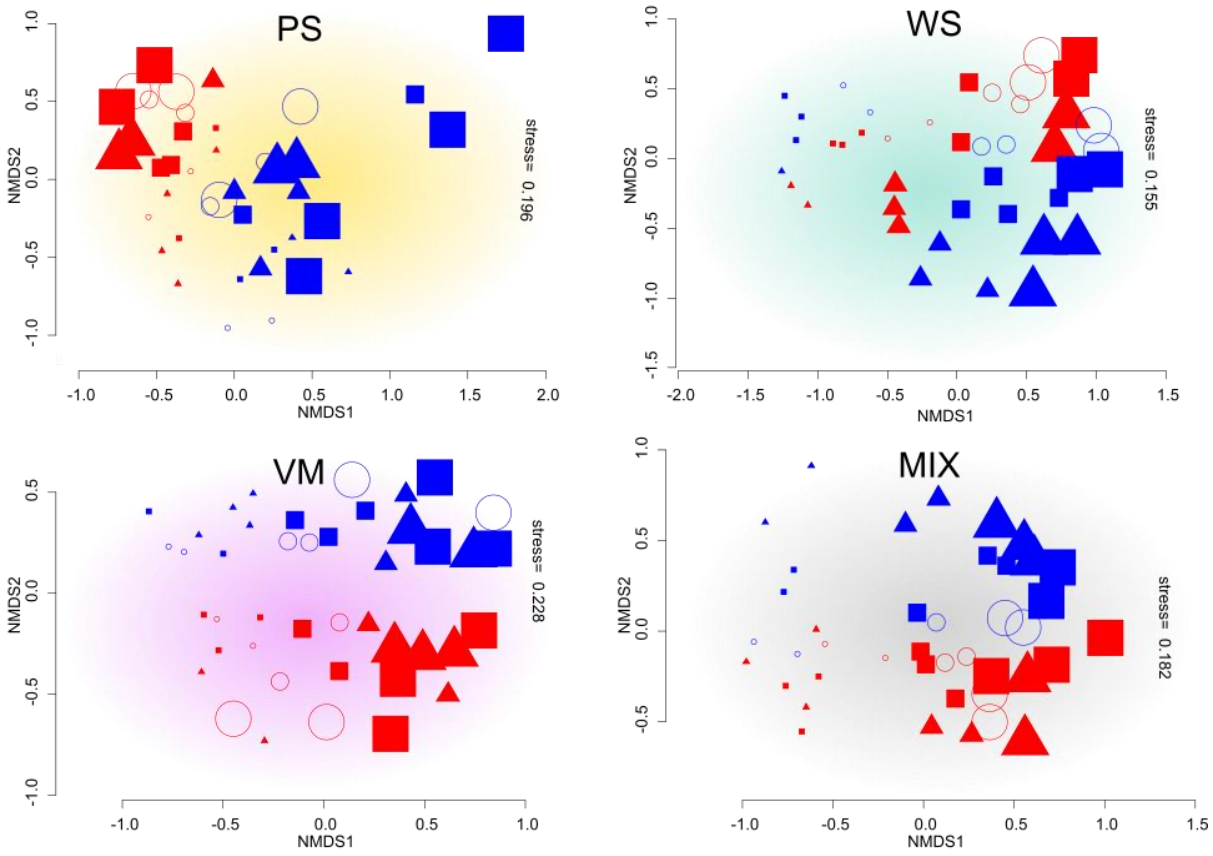

Figure 4: 2D NMDS diagrams showing OTU-level compositional changes of the microbiomes derived from the different inocula during the selection assay depend on host and medium type. Symbol colour represents medium (red is high; blue is low), symbol shape host type (circle: no diatom; square *C. closterium*; triangles *S. robusta*), symbol size increases with cycle (cycle I → III → VI).

Bacterial diversity (Shannon diversity) did not change significantly during the selection assay ($p>0.05$). The dissimilarity (Bray-Curtis distances), at the OTU level, between all bacterial communities on the other hand did increase over the growth cycles. This coincided with a divergence of the replicates over the cycles: the Bray-Curtis distances increased on average 0.05 per cultivation cycle ($p=0.005$). The combined explanatory power of the inoculum, medium and diatom species decreased over the cycles: from 51% after the first cycle to 21% after the sixth cycle. The inocula remained the most important variable over all the growth cycles (adj. $R^2_{\text{cycle I}}=0.45$; adj. $R^2_{\text{cycle III}}=0.29$; adj. $R^2_{\text{cycle VI}}=0.18$), explaining approximately seven times as much of the variance as either medium or diatom species. After the first cycle, the medium was twice as important (adj. $R^2=0.06$) as the diatom species (adj. $R^2=0.03$), but after the third (adj. $R^2=0.04$ for both) and sixth cycle (adj. $R^2=0.02$ for diatom species & adj. $R^2=0.03$ for inoculum) both had a comparable influence on the bacterial communities (Figure 4).

Overall, diatom species, medium and inocula had a strong influence on the bacterial communities (PERMANOVA: $p\leq0.0001$ for all cycles). The influence of these factors co-depended on the other factors as all interaction terms between the factors tended to be significant (PERMANOVA: $p<0.05$ for all cycles). Likewise, the species, medium and cycle were significant ($p<0.0015$) for each inoculum separately.

The growth of the hosts in the selection assay was initially strongly repressed compared to the axenic controls (Figure 5). While growth recovery took place in subsequent growth cycles in nearly all treatments, the recovery rate strongly depended on diatom species, inoculum and medium type (Supplementary Figure 4a). Growth recovery was faster for *S. robusta* than *C. closterium* and in high versus low resource medium (Figure 5). The VM inocula generally allowed for a faster growth recovery than the WS ones, and this effect was more pronounced for *S. robusta* than *C. closterium.* The inoculum effect strongly interacted with diatom species and medium type. For instance, the growth recovery of *S. robusta* in the high resource medium was the fastest with the MIX inocula whilst in the case of *C. closterium*, it was the slowest. In the final growth cycle of the selection assay, just over half (51.7%) of the diatom cultures grew faster with bacteria than without: most of these were *S. robusta* cultures (76.9% compared to 31.3% of the *C. closterium* cultures).

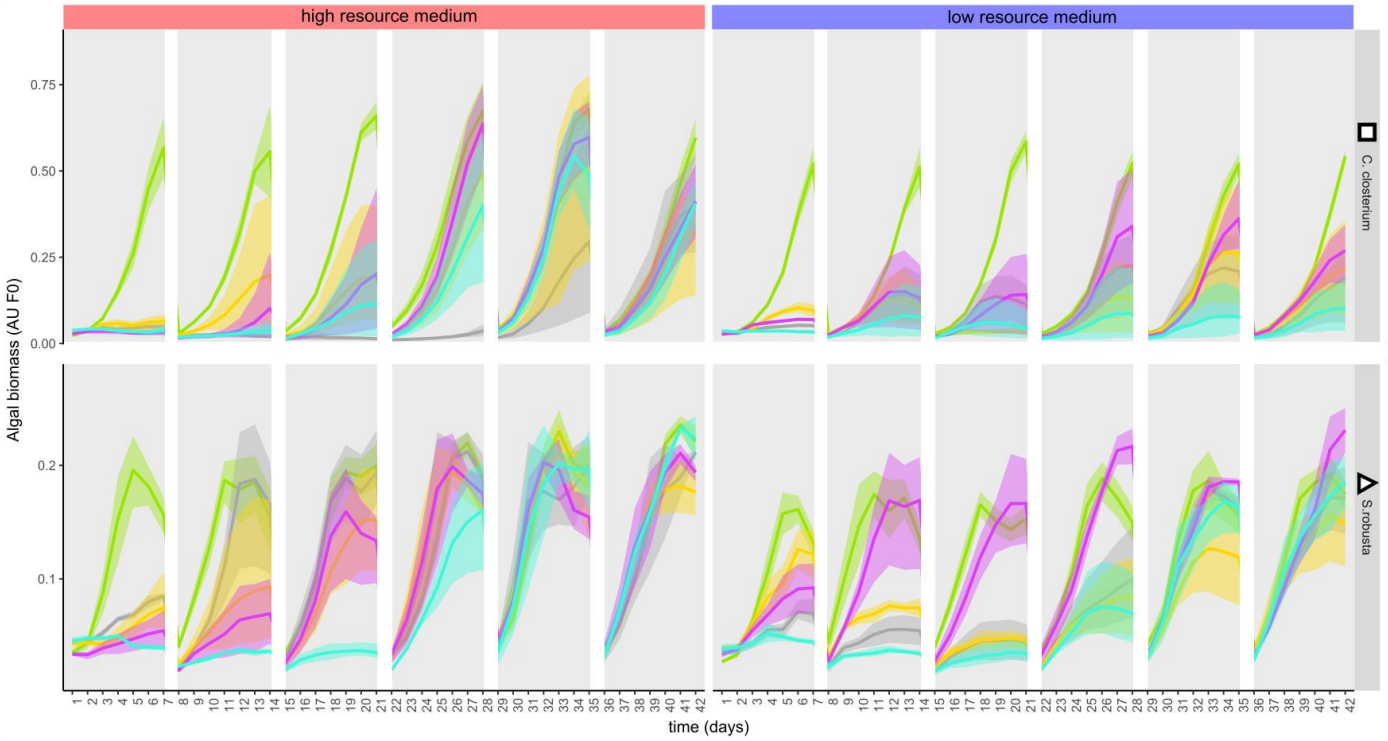

Figure 5: Growth recovery of the diatoms during the selection assay. Growth curves (based minimal fluorescence F_0) for each diatom species and medium type for the six consecutive growth cycles of the selection assay. The different colours of the growth curves represent the different inocula (cyan for WS, purple for VM, yellow for PS, grey for mixture of the three sources). The green curves show the growth of the axenic controls. Shading shows the variation between replicates.

Fitness assay

Diatom growth was again reduced in the first growth cycle of the fitness assay (Figure 6). The extent of growth inhibition depended foremost on the host species and medium type, but also on the prehistory of the bacterial community (Supplementary Figure 4b). The effect of the previous (selection assay) host and medium type and of the different inocula on diatom growth was still strong. In addition, the effect of the primary host on the growth of the secondary host was dependent on how both were related. For both diatom species, inoculum, the relation between primary and secondary host, the current medium and its relation to the previous medium were significant (mixed linear model: $p<0.05$), either as such and/or in interactions between terms. All interactions terms were highly significant ($p<0.0001$) as well.

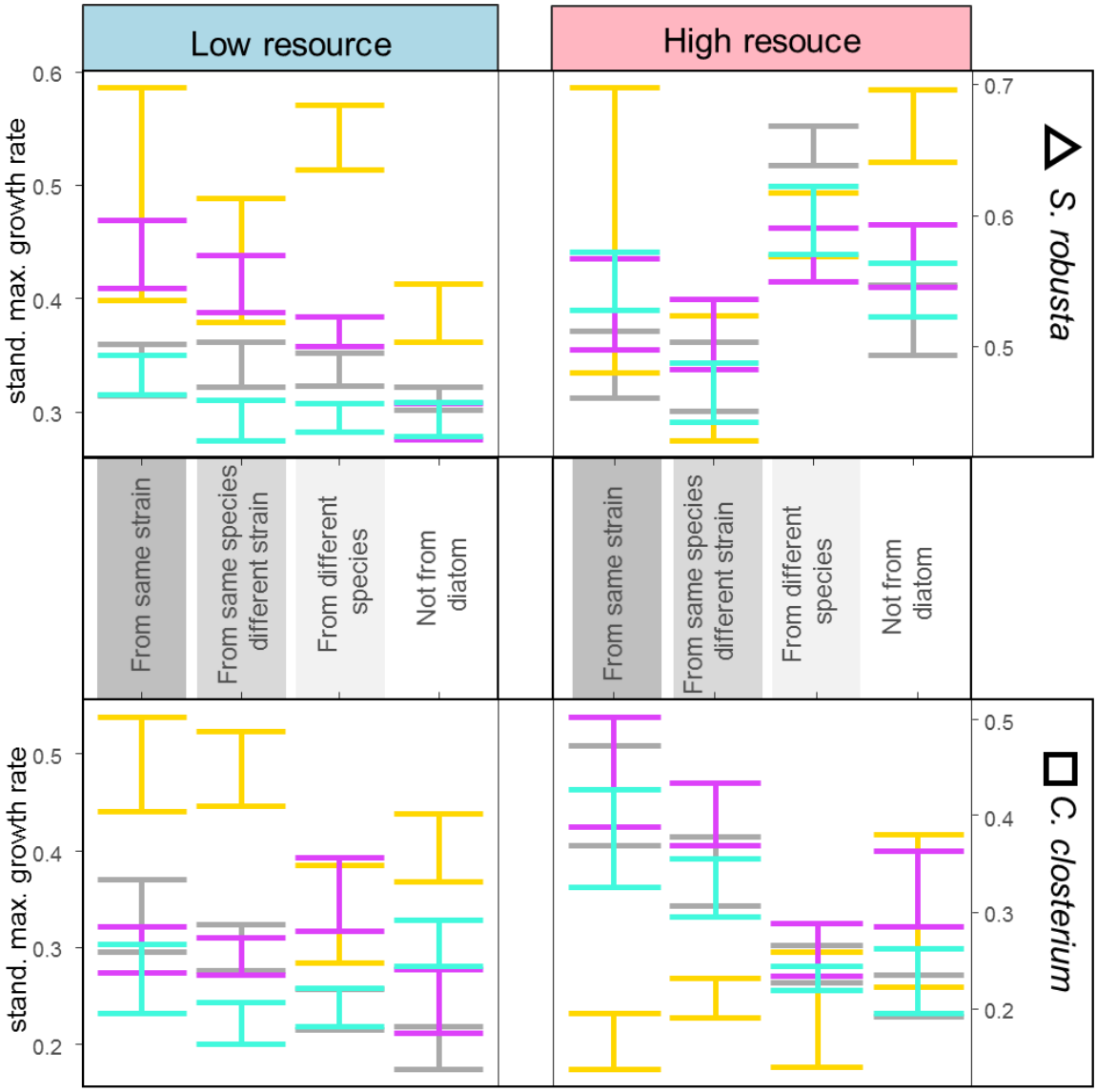

Figure 6: diatom growth responses towards its own and foreign microbiomes. The growth of the diatoms in the first cycle of fitness assay is shown for both diatom species and media. Since the diatom growth is standardised to the growth of axenic control, values below one are indicative of growth inhibition by the bacteria. The sources are indicated by different colours (cyan for WS, purple for VM, yellow for PS, grey for MIX). The relation between current and previous host affects the diatom growth differently depending on host, medium and source.

During the first cycle, bacterial communities originating from VM were least growth-inhibiting if the host was the same as in the selection cycle, but only for *S. robusta* in low resource medium and *C. closterium* in high resource medium. This effect diminished with decreasing phylogenetic relatedness to the secondary host. No (or even an opposite) effect was apparent for *S. robusta* in high resource medium and *C. closterium* in low resource medium (Figure 6). Similar trends were observed for the treatments with bacteria derived from WS and MIX inocula with *C. closterium*. Bacterial

communities derived from PS inocula showed no consistent patterns with respect to the relatedness between the primary and secondary host at all (Figure 6).

Despite not being identified as an important variable by the random forest model, the relationship between the selection and fitness assay medium impacted the bacterial effect on diatom growth in the first week of the fitness assay. If bacteria from the VM inoculum were previously cultured in the same medium, diatom growth was generally higher (Figure 7). For WS and MIX bacteria, diatom growth was generally higher if they had previously been cultured in a high resource medium. Bacteria from the PS inoculum were generally less growth inhibiting if they had previously been cultured in a different medium

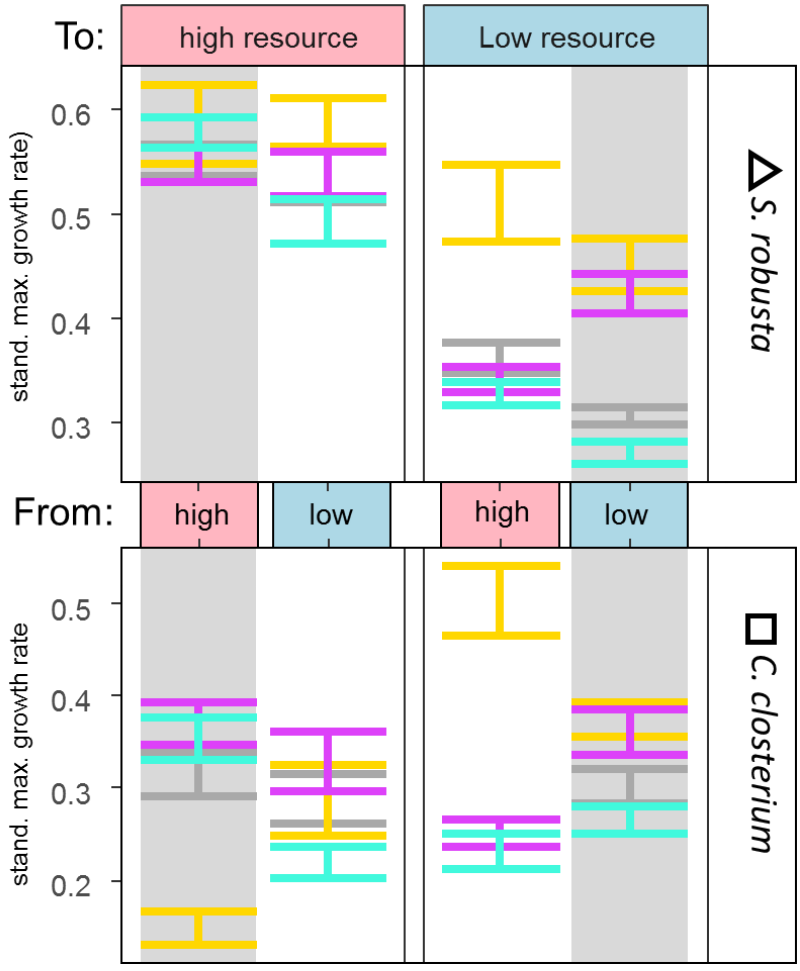

Figure 7: Diatom growth reduction by sympatric bacteria was less pronounced if the selection and fitness assay media were the same. Diatom growth (standardized to growth of axenic control) in the fitness assay medium in relation to the selection assay medium for both diatom species during the first cycle of the fitness assay. The original inocula are indicated by different colours (cyan for WS, purple for VM, yellow for PS, grey for MIX).

In the second and final growth cycle of the fitness assay, diatom growth was overall higher than in the first cycle (Supplementary figure 5). Growth of *S. robusta* in most of the cocultures was enhanced with respect to the axenic controls. In contrast, growth of *C. closterium* in the cocultures was still suppressed when compared to the controls. The difference between the two species was most pronounced in the low resource medium. Effects of primary host and media, that were observed in the first growth cycle, were no longer observed in the second growth cycle.

Discussion

Our experimental data show that microbiome assembly from widely different natural inocula was host and medium specific, and could contribute to enhanced growth of the host diatom. Diatom growth was strongly inhibited upon initial exposure of the diatoms to the bacteria. This is in contrast to what has been reported earlier with planktonic diatoms (Grossart et al. 2005). We argue that these differences are likely due inherent differences between planktonic and benthic bacterial communities. In a plankton environment nutrients tend to be more limiting and direct interactions between microorganisms are rarely beneficial in contrast to a benthic environment where there is a tight physical coupling between organisms (Stal et al. 2019). As anticipated (Rivett et al. 2016), the assembly process coincided with a recovery of diatom growth which is probably indicative for the gradual decrease of competition between the bacteria and their algal host (Ghoul & Mitri 2016). The growth recovery of the diatoms coincided with a compositional change of the bacterial community, and in particular a decrease of *Pseudomonas* spp., for the plant soil derived bacterial communities, and *Arcobacter* spp, for all other communities. These bacteria were largely replaced by Rhizobiales and Rhodobacteriales respectively. This shift might have been directly driven by the host (Foster et al. 2017) or more indirectly through the bacteria (Bauer et al. 2018), but either way, the result was beneficial for the host. Additionally, we cannot exclude micro-evolutionary changes to have taken place within six weeks of coexistence (Fiegna et al. 2015), such as the loss of bacterial virulence genes (Ochman & Moran 2001) but also more intricate adaptations between bacteria and host are possible (King et al. 2016; Mikonranta et al. 2015).

Despite the six week old microbiota being generally beneficial or commensal to the growth of the their primary host, addition of these ‘selected’ bacterial communities to a new, secondary host always resulted in an initial decrease of the host growth rate,

even if this host was genetically identical to the previous one. This shows that the interactions between diatom hosts and bacteria are indeed reciprocal and need time to establish. This may be related to changes in exudate production by the diatoms (Brucker et al. 2011; Buhmann et al. 2016), which in turn influence bacterial behaviour (Haynes et al. 2007; Tada et al. 2017). Similar dynamics were previously reported for another microalga (Seyedsayamdost et al. 2011), illustrating how an alteration in the physiological state of one of the partners can shift the balance between cooperation and competition.

The extent of growth rate decrease of the secondary host was variable and depended not only on the secondary host but also on its relation to the primary host. Only in the case of bacteria, sympatric to their secondary host, did competition with the secondary host decrease as a function of the phylogenetic similarity to the primary host. Whilst this was not always the case, such a pattern was never observed for allopatric bacteria. It is possible that the naturally co-occurring bacteria possessed the appropriate recognition mechanisms (Amin et al. 2012) to detect and engage with their host and the allopatric bacteria did not. These findings demonstrate that the host specificity of the microbiome does not consistently benefit its host and suggest that coadaptations between host and bacteria might be relevant to recognize suitable partners and avoid competition.

There were marked differences between the two diatom species in their response to bacteria. *S. robusta*, tended to grow better with bacteria than without once the interactions with its bacteria had been established. The growth of *C. closterium* however rarely exceeded that of the axenic control, indicative for the persistence of competition between diatom and bacteria. The decrease of competition with increased host similarity, as observed in the fitness assay, only occurred in the high resource medium for *C. closterium* whilst for *S. robusta* this was only found with the low resource medium. These differences might represent different strategies of the species towards bacteria, similar to what has been observed in plants (Lekbeg et al. 2018). Whereas *S. robusta* likely depends on bacteria for certain metabolites (Amin et al. 2012), which would indeed be more noticeable in a more deplete medium, *C. closterium* might be ecologically equivalent to fast-growing plants (Lekberg et al. 2018) and thereby more likely to compete with bacteria for nutrients. In a nutrient rich

medium, the competition might be relaxed, making way for more beneficial interactions.

The influence of the two media types on the bacterial community composition confirms the previously reported importance of the environmental conditions on the assembly process (chapter II). In contrast to what was expected, interaction specificity between bacteria and their host were not necessarily more pronounced in the low resource medium. Rather, sympatric bacteria tended to have a less negative effect on the fitness of their secondary host if the medium was unaltered, regardless of whether that was a high or a low resource medium. Allopatric bacteria tended to show the opposite response: they were more competitive to their secondary host if they had previously been cultured in the same medium. For both plants (Lau & Lennon 2012) and macroalgae (Dittami et al. 2015), it has also been reported that the host was more fit when its conditions matched the historical conditions of its microbiome. Whether the bacteria might aid their host to persist in different environments as was proven for these studies remains to be seen.

The bacteria coming from a plant soil showed comparable dynamics during the assembly process as the marine bacterial inocula, notwithstanding the fact that they were cultivated with marine diatoms in a marine medium. These findings illustrate how, at least to some extent, interactions between bacteria and a diatom can result in a microbiome independent of their prehistory. This relatively extreme example of ecological fitting (Agosta & Klemens 2008) underscores the remarkable metabolic flexibility of bacteria (Garcia et al. 2017). The prominent role of *Pseudomonas*, mirroring that of *Arcobacter* in the more natural situation is in accordance with the versatile metabolic capacity and broad potential for adaptation to fluctuating environmental conditions in this genus (Silby et al. 2011). *Pseudomonas* is also commonly reported in microbiota of plants (Berg & Smalla 2009), whereby it likely has several useful adaptations to interact with diatoms. In the absence of Rhodobacteriales, a group which is considered to be particularly well-adapted to interact with marine organisms (Luo & Moran 2014), Rhizobiales increased their abundance. Both alphaproteobacterial orders are prominent members of plant and algal associated microbiota respectively (Ramanan et al. 2016; Erlacher et al. 2015). Nonetheless, if the plant associated bacteria were challenged with marine bacteria, the plant associated bacteria were rapidly outcompeted, showing that, although they

were able to take up niches when available, they were not as well adapted to them as the marine bacteria were.

The influence of the host on the assembly process was surprisingly small. Notwithstanding the significant influence of the host on the composition of the bacterial community and the effect of this community on the diatom growth, the communities in the diatom free controls resembled those with a host well. The limited influence of the hosts puts what is generally considered a diatom-associated microbiome (Amin et al. 2012) in a new perspective. Since most studies that have characterised diatom-associated communities have worked with diatoms in cultures rather than *in situ* cells (e.g. Ajani et al. 2018; Schäfer et al. 2002), much of what has been considered diatom-associated bacteria might merely reflect the cultivation conditions itself and not the presence of the algal host. In general, the extent of host control on its microbiome remains highly debated for many eukaryotic organisms (Foster et al. 2017) and our results also warrant further investigation for the case of diatoms and their associated bacteria.

Conclusion

These results show that the interactions between bacteria and diatoms are reciprocal and result in algal microbiomes specific to both environment and host species. Specific algal microbiomes were also observed with bacteria not naturally occurring with their host and thus specificity does not require prior coadaptations between bacteria and their host. However, as only naturally co-occurring bacteria tended to show reduced competition with their host if grown in conditions similar to what they previously experienced, coadaptations do seem to be a requirement for the specificity of the microbiomes to be beneficial to the host. The remarkable flexibility of the host-microbiome relation and the prevailing effect of the cultivation conditions themselves, observed in this study, warrant further investigation.

Acknowledgements

The work of W. Stock was funded by the Fund for Scientific Research – Flanders (1115717N; FWO-Flanders, Belgium). This research was supported under the BIO-Tide project, funded through the 2015-2016 BiodivERsA COFUND call for research proposals, with the national funders BelSPO, FWO, ANR and SNSF. The research leading to results presented in this publication was carried out with infrastructure funded by EMBRC Belgium - FWO project GOH3817N. E.

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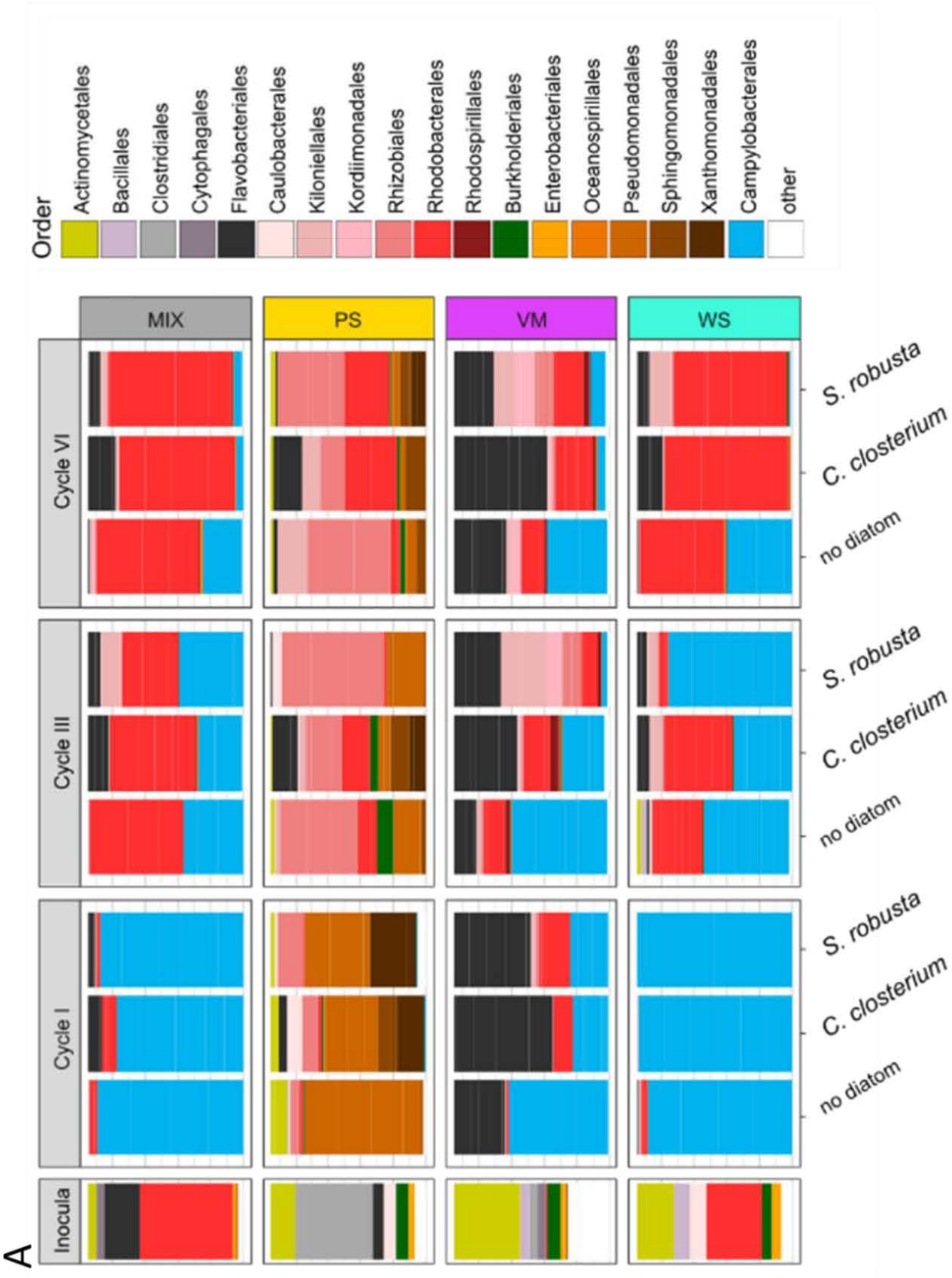
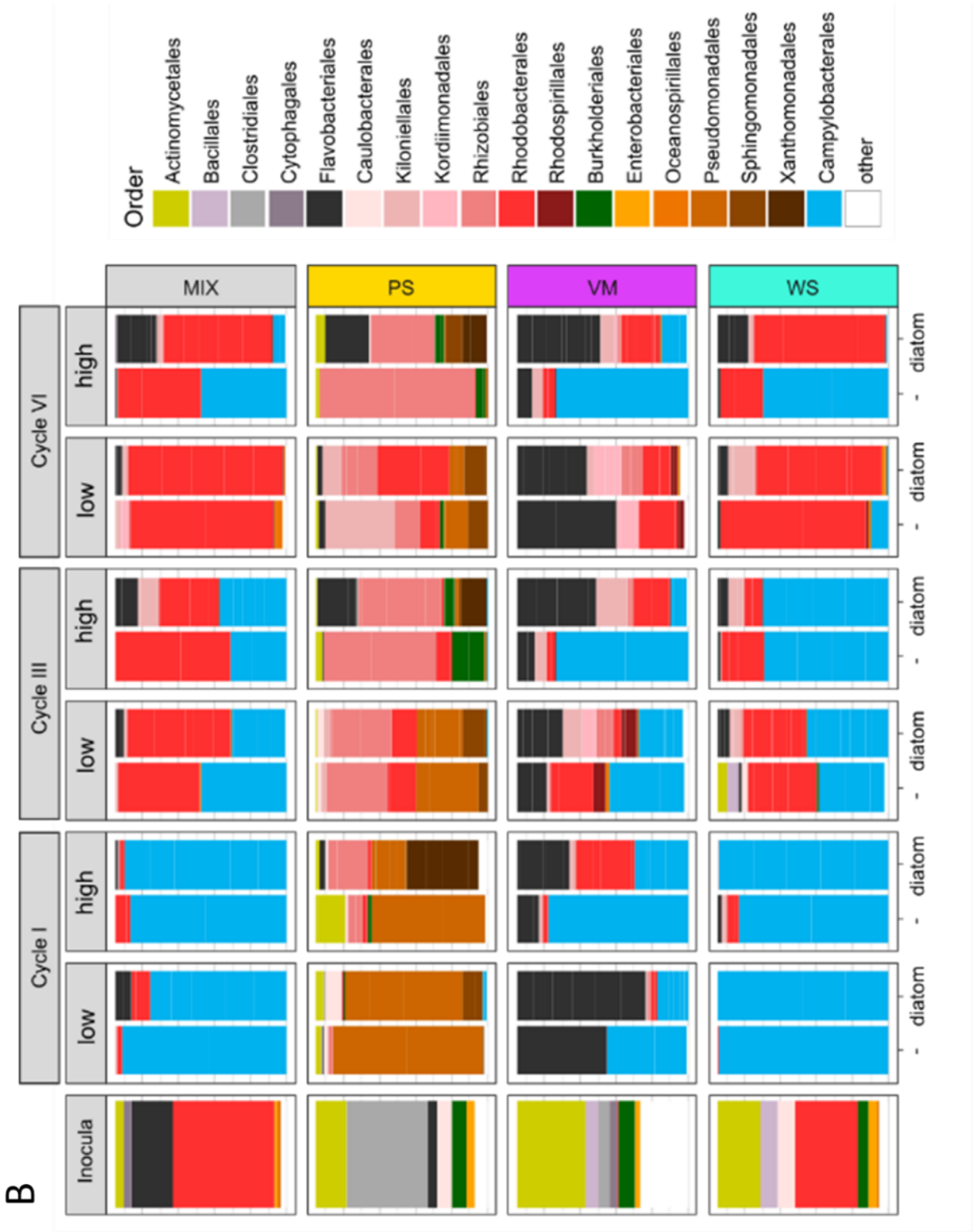
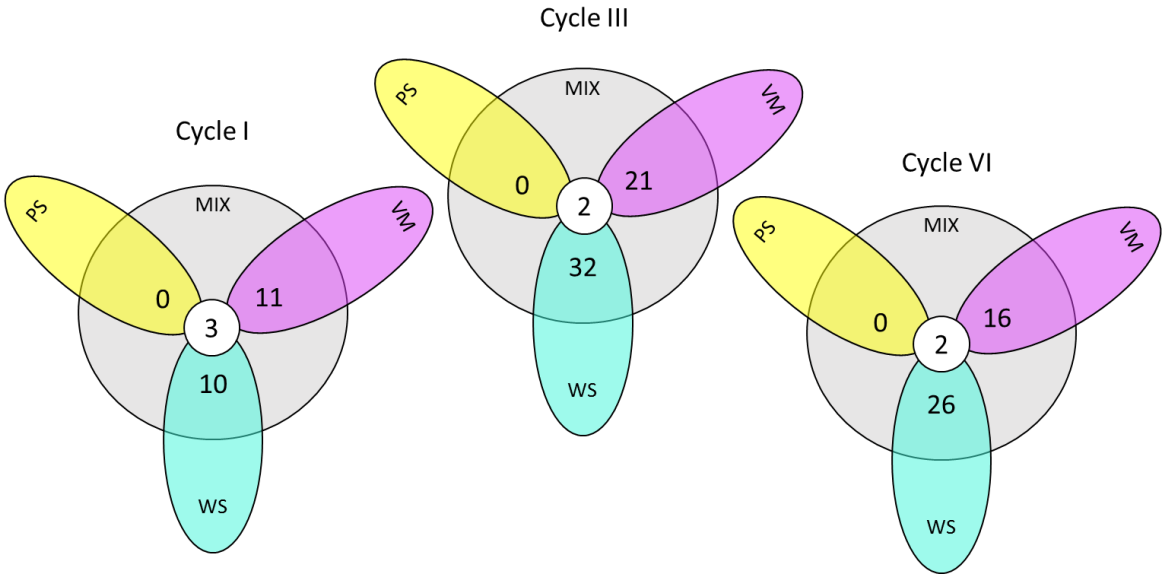
Supplementary material

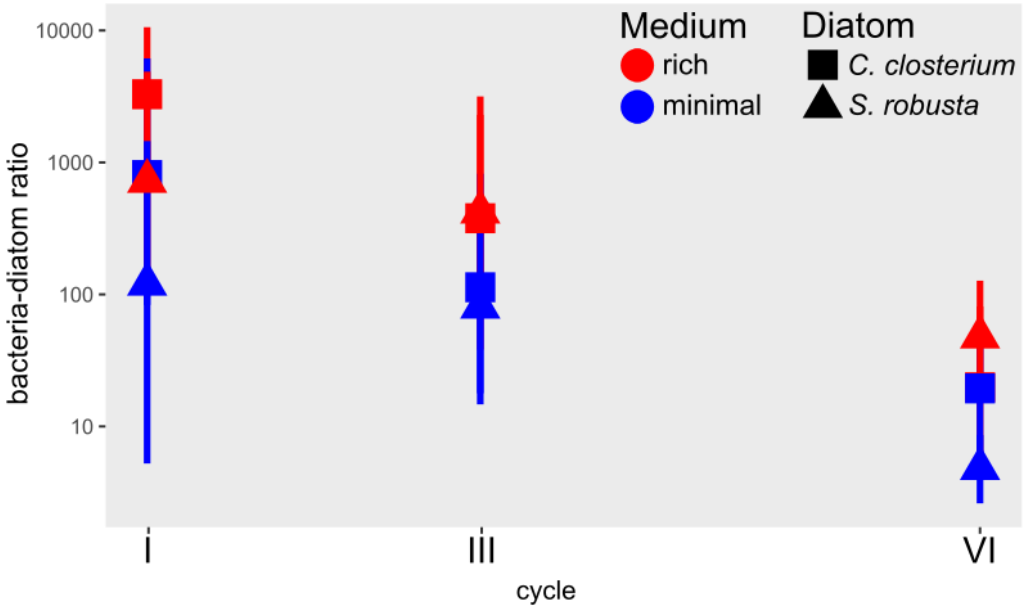




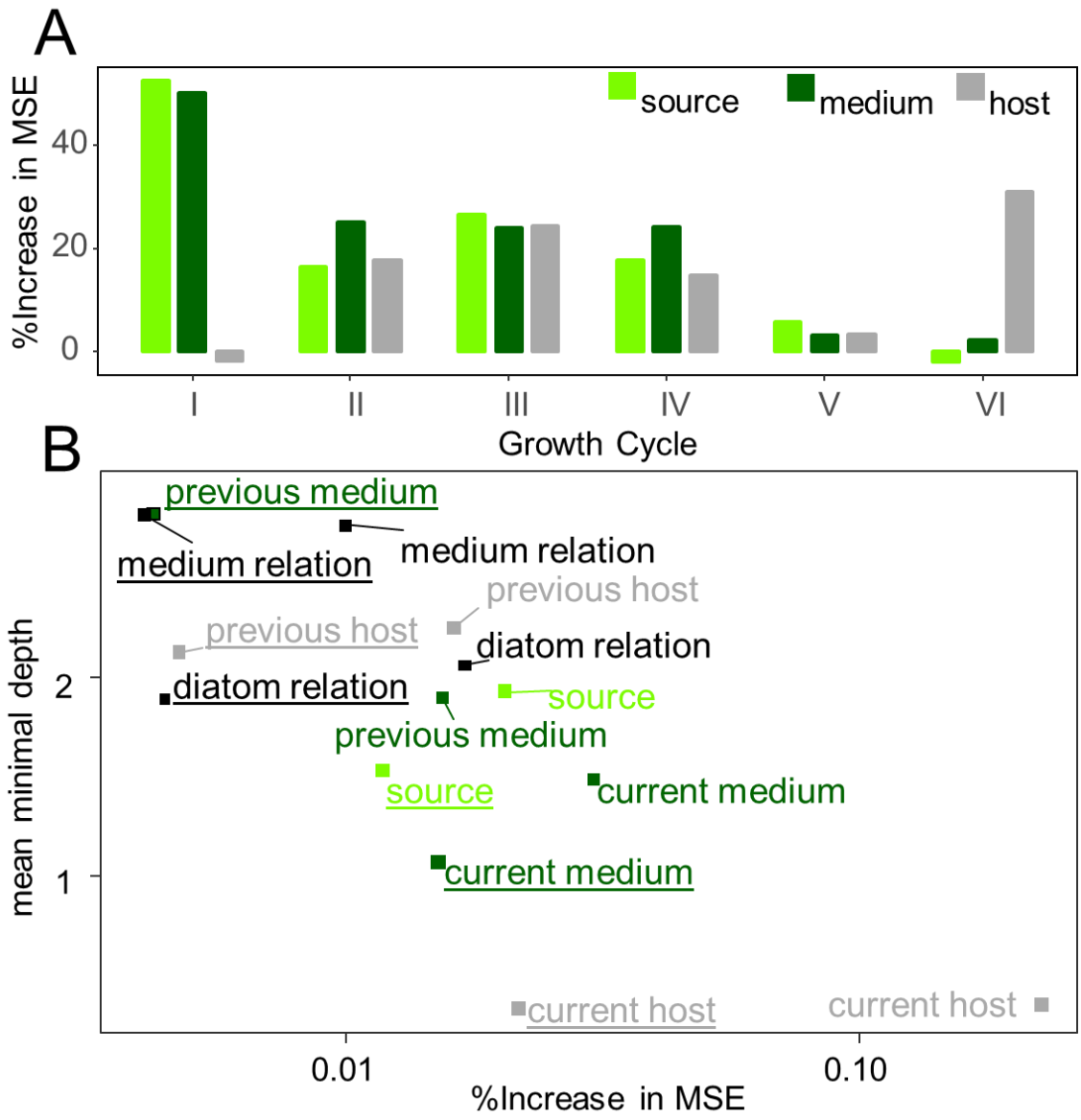
Supplementary Figure 1: Compositional changes in microbiomes during the selection assay. Average 16S rDNA based relative composition of the bacterial communities at the order level in the original inocula and after growth cycle I, III and VI. (A) shows the differences between the diatom treatments (averaged over both media types) and (B) between medium types (averaged over the *S. robusta* and *C. closterium* treatments).



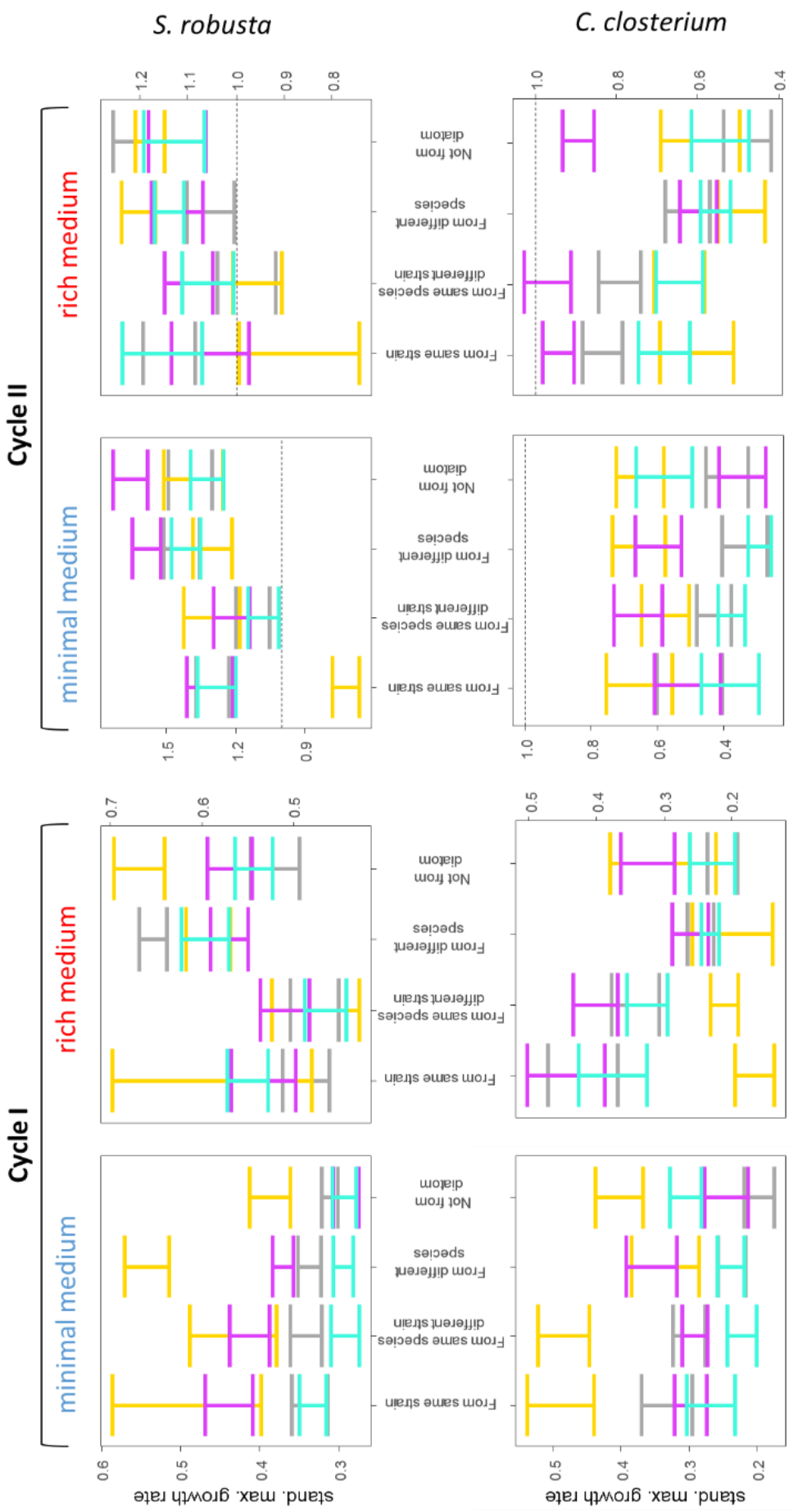
Supplementary Figure 2: The mixed microbiomes shared only the most common bacterial OTUs with the plant soil microbiomes. For first, third and sixth cycle of the selection assay, the number of OTUs shared with the MIX microbiomes is shown for the different bacterial sources. The central node shows the OTUs shared between all microbiota.



Supplementary Figure 3: The relative abundance of bacteria in relation to the diatoms declined during the selection assay. The ratio of bacteria to diatoms, calculated as the number of reads assigned to bacteria divided by the number of reads assigned to diatom chloroplasts, for different growth cycles. Note that the y-axis is logarithmic.



Supplementary Figure 4: The importance of the different elements for the diatom growth response as determined by the random forest analyses. The influence of the medium, host type and inoculum on the random forest model is shown for every growth cycle in the selection assay in the upper panel (A). For the fitness assay, secondary host and medium, inoculum, but also the primary host and medium and the relation between previous and current host and medium conditions are influential(B). The underlined variables relate to the first growth cycle of the fitness assay. The percentage increase in mean squared error (MSE) show the increase of MSE when the variable is randomly permuted by another variable in the random forest model, the higher the increase, the more important the permuted variable. The mean minimal depth shows the average depth that the variable first appears in the trees of the random forest, the lower, the more important it is.



Supplementary Figure 5: Changes in diatom growth responses to own and foreign microbiomes in cycle I and II of the fitness assay for both diatom species and medium types. Diatom growth is standardised to the growth of axenic control, hence values below one (dotted line) represent growth inhibition. The original inocula (selection assay) are indicated by different colours (cyan for WS, purple for VM, yellow for PS, grey for MIX).

Chapter V: Diatom-bacteria interactions modulate the composition and productivity of benthic diatom biofilms

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Diatom-bacteria interactions modulate the composition and productivity of benthic diatom biofilms

Published as: Koedooder, C., Stock, W., Willems, A., Mangelinckx, S., De Troch, M., Vyverman, W., & Sabbe, K. (2019). Diatom-bacteria interactions modulate the composition and productivity of benthic diatom biofilms. *Frontiers in Microbiology*, 10, 1255.

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Abstract

Benthic diatoms are dominant primary producers in intertidal mudflats and constitute a major source of organic carbon to consumers and decomposers residing within these ecosystems. They typically form biofilms whose species richness, community composition and productivity can vary in response to environmental drivers and their interactions with other organisms (e.g. grazers). Here, we investigated whether bacteria can affect diatom community composition and *vice versa*, and how this could influence the biodiversity-productivity relation. Using axenic experimental communities with three common benthic diatoms (*Cylindrotheca closterium*, *Navicula phyllepta* and *Seminavis robusta*), we observed an increase in algal biomass production in diatom cocultures in comparison to monocultures. The presence of bacteria decreased the productivity of diatom monocultures while bacteria did not seem to affect the overall productivity of diatoms grown in cocultures. The effect of bacteria on diatom growth, however, appeared to be species-specific, resulting in compositional shifts when different diatom species were grown together. The effect of the diatoms on the bacteria also proved to be species-specific as each diatom species developed a bacterial community that differed in its composition. Together, our results suggest that interactions between bacteria and diatoms residing in mudflats are a key factor in the structuring of the benthic microbial community composition and the overall functioning of that community.

Introduction

Intertidal mudflats, found predominantly along estuaries and sea inlets, are highly productive ecosystems (Underwood and Kromkamp, 1999). Their productivity is in part due to benthic microalgal biofilms found on the surface sediments (Decho, 2000; Hochard et al., 2010), with benthic diatoms often being one of the dominant primary producers (Admiraal et al., 1984; Bolhuis et al., 2013; MacIntyre et al., 1996; Underwood and Kromkamp, 1999). These biofilms strongly modulate nutrient fluxes (Cook et al., 2009; Hochard et al., 2010) and provide copious amounts of autochthonously fixed carbon to successive trophic levels within the mudflat (Moerdijk-Poortvliet et al., 2018). The productivity of benthic biofilms, dominated by diatoms, depends on the diatom community composition and structure (Brotas and Plante-Cuny, 2003; Underwood et al., 2005) which in turn is dependent on a combination of both abiotic and biotic factors. Although the variable distribution of diatoms along estuarine gradients can partially be explained by the species-specific tolerances of diatom species for environmental conditions such as salinity and nutrient concentrations (Desianti et al., 2017; Forster et al., 2006; Ribeiro et al., 2013; Sawai et al., 2016; Thornton et al., 2002), biotic factors, such as competition and niche differentiation between different diatom species (Vanelslander et al., 2009) as well as the presence of bacteria (D'Costa and Anil, 2011), can also affect the diatom community structure and composition. There is as yet, however, no overall consensus on the exact relation between primary productivity and algal biodiversity of intertidal benthic biofilms. The biodiversity-productivity relation was shown to vary across sites (Forster et al., 2006) and could be either negative or positive (Forster et al., 2006; Vanelslander et al., 2009). Negative biodiversity effects on productivity are often attributed to competition or chemical interference (i.e. allelopathy) while positive biodiversity effects are the result of selection and/or complementarity effects (Loreau and Hector, 2001). A positive selection effect occurs due to the dominance of highly productive species while a complementarity effect increases productivity by enhancing the use of the available resources as a result of positive interactions or niche differentiation. This study investigates the various interactions taking place within a benthic diatom community and whether the presence of bacteria impacts the community regarding species composition and productivity and *vice versa*.

Due to the confined space within the matrix of marine benthic biofilms, interactions between the diatoms and other organisms becomes inevitable (Clarke, 2016). The excretion of exopolymeric substances (EPS) by diatoms, for example, serves as an important carbon source that can be utilized by the residing benthic organisms (Bellinger et al., 2009; Middelburg et al., 2000) including heterotrophic bacteria (Durham et al., 2015; Middelburg et al., 2000; Taylor et al., 2013) as well as other diatom species (Vanelslander et al., 2009). Since the composition of diatom exudates is both species-specific and dependent on environmental conditions (Bohórquez et al., 2017; Durham et al., 2015), EPS has been suggested to play a major role in determining bacterial community composition and diversity (Agogue et al., 2014; Haynes et al., 2007; Mühlenbruch et al., 2018; Wear et al., 2015). Indeed, studies have shown that diatom species can harbour different associated bacteria (Behringer et al., 2018; Doiron et al., 2012; Grossart et al., 2005; Schafer et al., 2002) and that bacterial community composition in intertidal mudflats strongly co-varies with the composition of the microphytobenthos (Bolhuis et al., 2013; Decleyre et al., 2015; Lavergne et al., 2017).

In turn, the associated bacteria have also been shown to influence diatom growth rates as well as other life cycle features such as sexual reproduction (Amin et al., 2012; Cirri et al., 2018; Grossart, 1999). Despite the general view of heterotrophic bacteria as the primary remineralisers of (e.g. diatom-derived) organic matter (Azam, 1998; Taylor et al. 2013), releasing nutrients back into the environment, the bacterial community can also compete with diatoms for limited nutrients and resources (Amin et al., 2012; Grossart, 1999; Havskum et al., 2003; Thingstad et al., 1993). Other, more specific, interactions have also been observed, such as the ability of strains to produce vitamins (i.e. cobalamin) for auxotrophic diatoms (Croft et al., 2005; Haines and Guillard, 1974), as well as negative interactions such as the production of algicidal metabolites and the induction of diatom cell lysis (Furusawa et al., 2003; Paul and Pohnert, 2011). These effects can be highly specific, with some bacteria having the ability to stimulate the growth of one diatom whilst inhibiting the growth of another (Grossart, 1999; Jung et al., 2008; Paul and Pohnert, 2011; Sison-Mangus et al., 2014).

As many of the above-mentioned studies focussed on one-to-one interactions between diatoms and bacteria, they do not reflect the ecological complexity found in nature. Bigalke and Pohnert, (2019), for example, recently illustrated that a more

complex diatom community reacts differently to bacteria than the individual species did. While biotic and abiotic factors could promote the presence of a potentially harmful bacterial community (Marzinelli et al., 2018) the host-associated bacterial community can also play a role in its resistance and tolerance towards a new or stressful environment (Dittami et al., 2016). Studies like these, stress that the oversimplification of experiments can prevent further insight into relevant ecological interactions. Whether a bacterial community can influence community functioning in terms of productivity and the outcome of interspecific interactions amongst co-occurring biofilm diatom species has to date not been tested. Although D'Costa and Anil (2011) have shown that diatom community structure is related to bacterial community composition, it was not determined whether and how diatom-bacteria associations could affect changes in diatom productivity and interactions. Recent work on plants and macroalgae, suggests that feedback mechanisms between hosts and their specific bacterial community can ultimately determine the outcome of competition amongst host species (Dittami et al., 2016; Gribben et al., 2017; Hortal et al., 2017; Lekberg et al., 2018; Marzinelli et al., 2018).

Using different benthic diatom species grown at three diversity levels (monoculture vs 2- and 3-species cocultures) in the presence or absence of a natural bacterial inoculum, we investigated whether bacteria can change the structure and productivity of simple diatom communities. We hypothesize these interkingdom interactions are species-specific and that certain bacteria promote the growth of selected diatoms and *vice versa*. We therefore predict that the interactions between diatoms and bacteria will change the outcome of competition amongst the different diatom species, favouring the diatom species that benefit the most from the presence of bacteria. This, in turn, could further influence diatom productivity. Finally, we tested whether diatoms can also affect bacterial diversity, predicting an increase in bacterial diversity with increasing diatom diversity and productivity.

Material and Methods

Experimental setup

The marine benthic diatoms *Cylindrotheca closterium*, *Navicula phyllepta* and *Seminavis robusta* were obtained from the BCCM-DCG culture collection (Supplementary Table 1). The three strains were originally not isolated from the same location, but these three diatom species have been observed to co-occur, making them ecologically relevant for this study. Diatoms were cultivated in artificial sea water (ASW; Tropic Marin Bio-Actif Salt) that was enriched with 0.08 g/L $NaHCO_3$ and Guillard's Marine Water Enrichment Solution (F/2; Sigma) according to the manufacturer's instructions. Throughout the experiment, cultures were grown in 12:12h light-dark cycles using cool fluorescent white light (20-25 µmol photons/s/m²) and maintained at 18 °C.

Diatoms were made axenic (free of bacteria) by subjecting them to repeated antibiotic treatments. Every three days, the medium of the diatoms was refreshed and the next antibiotic treatment was given to the cultures. This process was repeated at least three times. Antibiotic mixes consisted of final concentrations of 100 µg/mL gentamycin, 500 µg/mL streptomycin and 100 µg/mL neomycin for *C. closterium*; and 500 µg/mL penicillin, 500 µg/mL ampicillin, 100 µg/mL streptomycin and 50 µg/mL gentamycin (Sigma Aldrich) for *N. phyllepta* and *S. robusta*. DAPI staining (Shishlyannikov et al., 2011) and plating on Difco Marine agar (BD) was used to confirm axenicity of the diatom cultures after the treatments.

Prior to the start of the experiment, antibiotics in the diatom cultures were washed away by repeated (3 times) refreshing of medium with new ASW three days after the last antibiotic treatment. The refreshed cultures were diluted to approximately 300, 100 and 85 cells/mL for *S. robusta, C. closterium* and *N. phyllepta*, respectively. Dilutions were based on prior experiments (data not shown), which showed that the coexistence between the diatom species required higher starting densities of *S. robusta.* At equal starting densities, *S. robusta* was rapidly outcompeted due to its lower growth rate.

The bacterial inoculum was obtained from intertidal surface mud collected at the Paulina polder, Westerschelde, NL (2 March, 2016 at 51°21'032"N, 3°43'574" E). This sample was taken one day before setting up the experiment. During this time, it was stored at 4 °C. On the day of the experiment, 20 mL of ASW was added to ±10 mL of

sediment, vortexed and then filtered (3 µm filter size) to separate the bacteria from larger eukaryotic organisms and particles. 1 mL of the bacterial suspension was frozen (-20 °C) for DNA extraction (see below).

The experiments were run in 24-well plates (Greiner Bio-one) with the addition of either 1 mL of bacterial inoculum (non-axenic) or 1 mL of ASW (axenic). 1 mL of diatom suspension was then added to obtain a total volume of 2 mL per well. Specifically, 1 mL of a single diatom, 2x0.5 mL of two diatoms or 3x0.333 mL of all three diatoms were added to obtain monocultures and 2- and 3-species cocultures, respectively. F/2 stock solution was supplemented to all wells so that a final nutrient concentration of F/20 (one tenth of F/2) was reached. All different combinations were run in triplicate (n=3), randomised across each plate and grown in the conditions stated above.

Daily biomass estimates were obtained using both pulse amplitude modulation (PAM) fluorometry (IMAGING-PAM M-Series Maxi version, Walz) and cell counts Appendix I). Minimal fluorescence (F_0) was measured (intensity:6, gain:3, frequency:1) daily, for a period of seven days. Photographs were taken of each well in triplicate (Nikon Elemental Camera DS-Fi2 with 10x magnification) using an inverted Axiovert 135 Zeiss microscope. Diatoms were manually counted from the pictures (Supplementary Figure 1) and averaged to obtain daily diatom cell counts.

After 1 week, 1 mL of medium was extracted from every well and stored at -20 °C for DNA analysis. DNA extractions were conducted according to Muyzer et al. (1993). The library prep and amplification of the 16S rRNA gene, using primers PA_ill and BKL1_III, were done according to D'Hondt et al. (2018). An artificially created mock community was included (Tytgat et al. 2016) to benchmark processing variables (Tytgat et al., 2016).

Data analysis and statistics

Diatoms

Growth curves were constructed from diatom cell counts and PAM fluorometry measurements (F_0) using Excel (Version 15.32). The maximal growth rate (μ_{max}) was defined as the highest observed growth rate during the exponential growth phase (the first four days as determined after inspection of each individual growth curve) of the experiment and calculated from the slope (LINEST function) after a log2 transformation for every three consecutive days using a moving window. This strategy

was applied to both F_0 and diatom cell counts. A two-way ANOVA (R-version 3.4.1) was used to test for differences in μ_{max} between diatom combinations and in the presence or absence of bacteria. The assumptions of ANOVA regarding homogeneity of variance and normality were respectively verified through a Levene and Shapiro-Wilk Test. Where appropriate, a Tukey HSD test was applied as post-hoc test.

Diatom productivity was defined as the increase in the diatom biovolume and calculated by multiplying the μ_{max} obtained from the cell counts with the cell biovolume calculated according to Hillebrand et al. (1999). Productivity was expressed in function of diatom biodiversity. Biodiversity effects were further partitioned into selection and complementarity effects according to Loreau and Hector (2001) in R (version 3.4.1). The partitioning of biodiversity effects was done separately for both axenic and non-axenic cultures.

Bacteria

The 300bp pair-end Miseq reads were joined and quality-filtered using PEAR v0.9.5. After primer removal, an operational taxonomic unit (OTU) table was constructed by clustering at a 3% divergence level (USearch8). Taxonomic assignment of OTUs was done using MOTHER (Version 1.35.1). Chimeras were removed using the internal check in Usearch whilst mitochondrial and chloroplast reads were removed based on taxonomic assignment. The OTU table was further processed in R (version 3.4.1). To remove potential cross-contamination and sequencing errors, read counts <5 and OTUs that were present in less than 3 samples were set to 0 (based on 16S mock community data). Relative abundances were derived from the constructed OTU table. Rare OTUs (<1% of number of reads/sample) were removed. A PERMANOVA was run using the Adonis function (Vegan Package 2.4-3) between all diatom combinations and treatments. Significance values were based on 1000 permutations. A constrained correspondence analysis (ANOVA-CCA, Vegan Package 2.4-3) was performed with different diatom combinations set as Boolean variables to identify the influence of the different diatom species on the bacterial community composition. Finally, a Simper Analysis (10000 permutations, Vegan Package 2.4-3) was run to identify the discriminating bacterial OTUs between the diatom monocultures. Bacterial diversity, expressed as both the predicted number of OTUs when rarefying all samples to the same depth and the Simpson's index, was also linearly expressed in function of both

diatom productivity and diatom-diversity. This procedure was separately repeated for the most diverse bacterial group, namely the Alphaproteobacteria.

Results

Diatom growth

In axenic conditions, *C. closterium* and *N. phyllepta* displayed comparably high μ_{max} in monocultures while *S. robusta* grew considerably slower (Figure 1, Table 1). The presence of other diatoms did not influence the μ_{max} of *C. closterium* ($p>0.05$) and *N. phyllepta* ($p>0.05$) but significantly increased ($p<0.001$) the μ_{max} of *S. robusta* (Figure 1a). The addition of a mixed natural bacterial inoculum to the monocultures significantly lowered the μ_{max} of *C. closterium* ($p=0.011$) and *N. phyllepta* ($p=0.007$), but not of *S. robusta* ($p>0.05$) (Figure 1a). In the non-axenic cocultures, the negative effect of bacteria on the growth of *N. phyllepta* and *C. closterium* seemed slightly alleviated by the presence of other diatoms (albeit not significantly), although the total cell numbers never exceeded those of their axenic counterparts (Figure 1b). The growth rate of *S. robusta* was again significantly higher ($p\leq0.003$) in mixed diatom cultures and in the presence of the two other diatom species, *S. robusta* was the fastest grower (Figure 1a).

These species-specific changes in growth rates due to the presence of bacteria resulted in compositional shifts in the diatom cocultures: the relative abundance of *S. robusta* was higher when bacteria were present, mainly at the expense of *C. closterium* (Supplementary Figure 4-5). Similar trends were observed for the μ_{max} derived from daily fluorescence measurements (Supplementary Figure 3, Supplementary Table 2) although PAM measurements did not allow separation of biomass increase between diatom species grown in cocultures. Altogether, changes in μ_{max}, from both cell counts (Figure 1a) or PAM measurements (Supplementary Figure 3) as well as the total biovolume (Supplementary Figure 5) indicate that, while the growth rates of *C. closterium* and *N. phyllepta* were significantly repressed in the presence of bacteria, *S. robusta* was left unaffected while its μ_{max} significantly improved in the presence of other diatom species.

Table 1: Statistical analysis of the maximum growth rates derived from individual cell counts for *S. robusta* [S], *C. closterium* [C], and *N. phyllepta* [N]. A two-way ANOVA was conducted testing the differences between the diatom combinations and the presence or absence of bacteria per diatom species. A post hoc test (Tukey Test) was conducted when significant differences were observed from the ANOVA. An asterisk marks results of significant value where * < 0.05, ** <0.01, and *** < 0.001.

Cell Counts					
C. closterium					
Two-way ANOVA	**Factor**	**DF**	**F value**	**p-value**	**significance**
	diatom combination	3	0.527	0.670	
	bacteria - axenic	1	8.250	0.011	*
	interaction	3	1.179	0.349	
		16			
Post hoc	**Conditions**	**Difference**		**p-value**	**significance**
	bacteria - axenic	-0.194		0.011	*
S. robusta					
Two-way ANOVA	**Factor**	**DF**	**F value**	**p-value**	**significance**
	diatom combination	3	20.784	<0.001	***
	bacteria - axenic	1	0.210	0.653	
	Interaction	3	1.554	0.239	
		16			
Post hoc	**Conditions**	**Difference**		**p-value**	**significance**
	[s] – [s+c]	-0.359		0.002	**
	[s] – [s+n]	-0.438		0.003	**
	[s] – [s+c+n]	-0.621		<0.001	***
	[s+n] – [s+c]	-0.078		0.770	
	[s+c] – [s+c+n]	-0.261		0.024	*
	[s+n] – [s+c+n]	-0.183		0.148	
N. phyllepta					
Two-way ANOVA	**Factor**	**DF**	**F value**	**p-value**	**significance**
	diatom combination	3	0.302	0.824	
	bacteria - axenic	1	9.257	0.008	**
	interaction	3	1.807	0.186	
		16			
Post hoc	**Conditions**	**Difference**		**p-value**	**significance**
	bacteria - axenic	-0.285		0.008	**

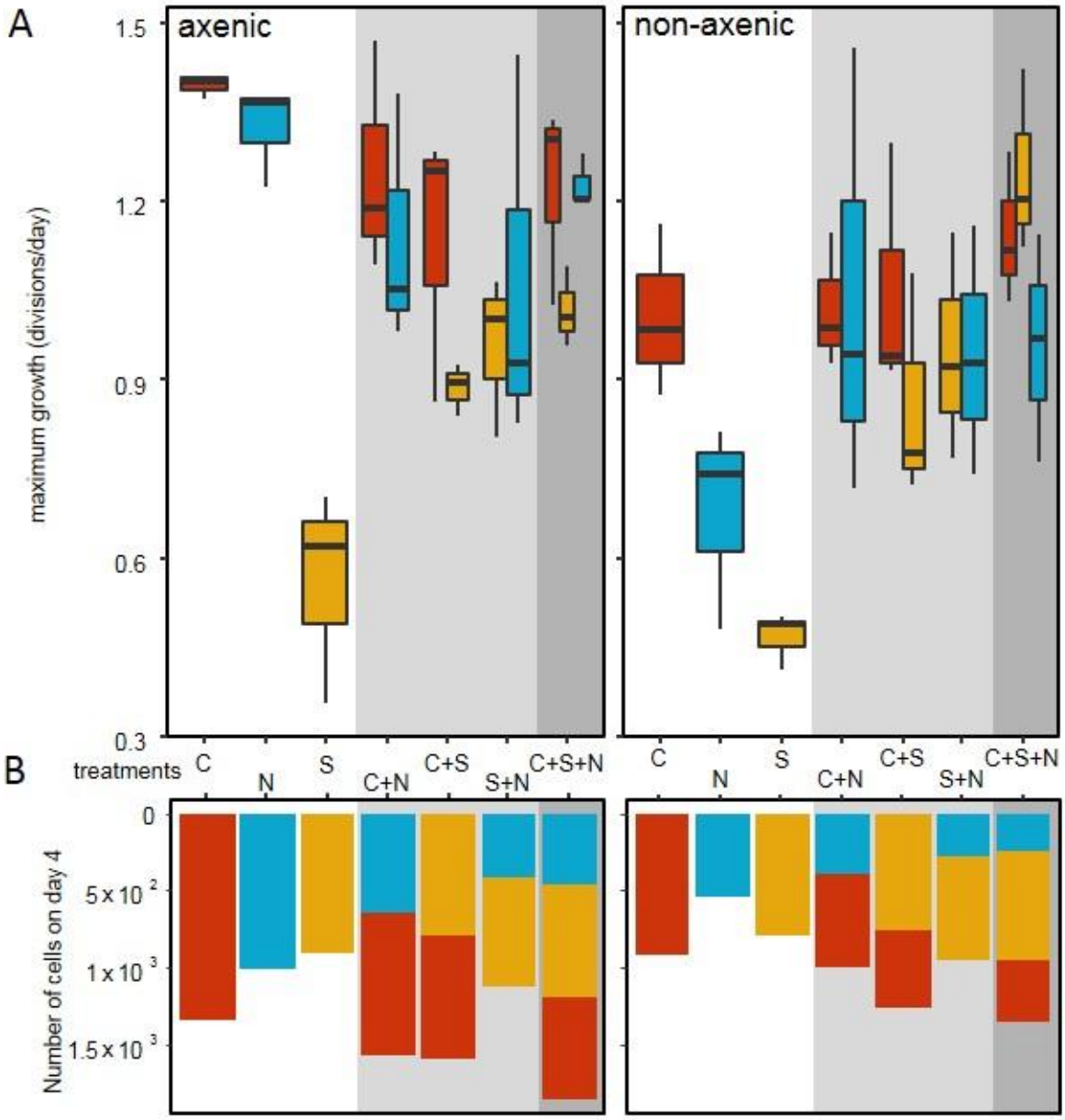

Figure 1: Bacteria cause a shift in diatom growth rates. (a) Maximum growth rates, derived from daily cell counts for three different diatom species: *C. closterium* ([C], red), *N. phyllepta* ([N], blue) and *S. robusta* ([S], yellow) in the presence (non-axenic) or absence (axenic) of bacteria. The white, grey and dark grey background respectively show diatoms grown either in monocultures, diatom pairs or the three diatom-species combined. (b) The number of diatom cells per well on the fourth and last day are shown using the same colour code as above.

Additive partitioning of biodiversity effects

We determined whether increasing diatom species richness had an impact on productivity, i.e. the algal biomass production, and whether this would change in the presence of bacteria (Figure 2). In general, productivity increased with increasing diatom diversity ($p<0.0001$) and the presence of bacteria steepened this relationship

(p=0.007; Figure 2a). This effect of bacteria can largely be attributed to a strong decrease in productivity in the diatom monocultures when exposed to the bacterial inoculum (p=0.045). In contrast, the bacteria have a reduced negative effect on the diatom species grown in the presence of other diatoms.

Analogous to the results obtained from the diatom growth rates, the productivity of *S. robusta* strongly increased in the presence of other diatoms under axenic conditions, while both *C. closterium* and *N. phyllepta* had lower productivity in the cocultures (Figure 2b). Adding bacteria switched the observed negative diversity effect in *C. closterium* and *N. phyllepta* to a positive diversity effect, with both species now showing higher productivity in the cocultures. This effect however was not very strong, and was always less pronounced in comparison to *S. robusta* (Figure 2b).

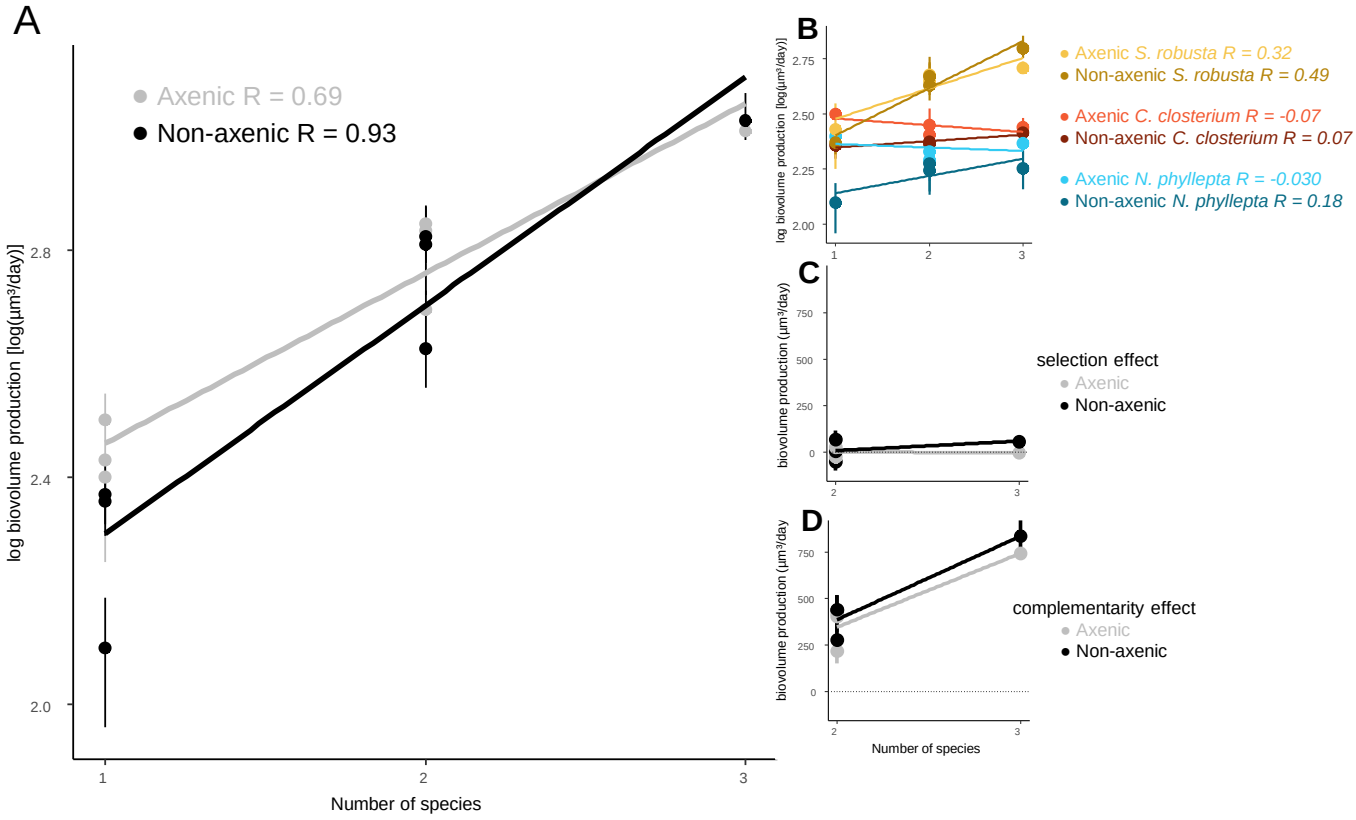

Figure 2: The presence of bacteria steepens the algal diversity-productivity relation. (a) Diatom biovolume production in function of species richness and its slope (m) in the presence (non-axenic, black line) or absence of bacteria (axenic, grey line). (b) Algal biovolume production per diatom species and its slope for each diatom: *S. robusta* (yellow), *C. closterium* (red) and *N. phyllepta* (blue). The presence or absence of bacteria are respectively depicted as dark- and light-coloured lines. The contribution of the selection and complementarity effects to diversity-productivity relation are respectively shown in (c) and (d) in the presence (black) or absence (grey) of bacteria.

The effect of diatom diversity on the productivity was further partitioned into selection and complementarity effects (Loreau and Hector, 2001) (Figure 2c and 2d). The observed increase in production ($p<0.0001$) can largely be attributed to positive complementarity effects, which further increased in the presence of bacteria ($p>0.05$). A positive but very minor selection effect on productivity was also observed in the presence of bacteria ($p>0.05$).

Bacteria community composition

After removing non-bacterial and potentially chimeric reads from the 16S rDNA high-throughput sequencing, 8724± 5505 reads per sample remained. These were assigned to 123 operational taxonomic units (OTUs). The sequencing depth was sufficient to cover most of the bacterial diversity present in the algal cultures (Supplementary Figure 2). The composition of the initial bacterial inoculum was dominated by Rhodospirillaceae (41.5% of the inoculum reads) (Figure 3). Following cocultivation with diatoms over a week, the bacterial communities became dominated by Rhodobacteraceae, Flavobacteriaceae and Campylobacteraceae both in terms of diversity (32, 18 and 8%, respectively) and relative abundance (68.6 and 14%, respectively).

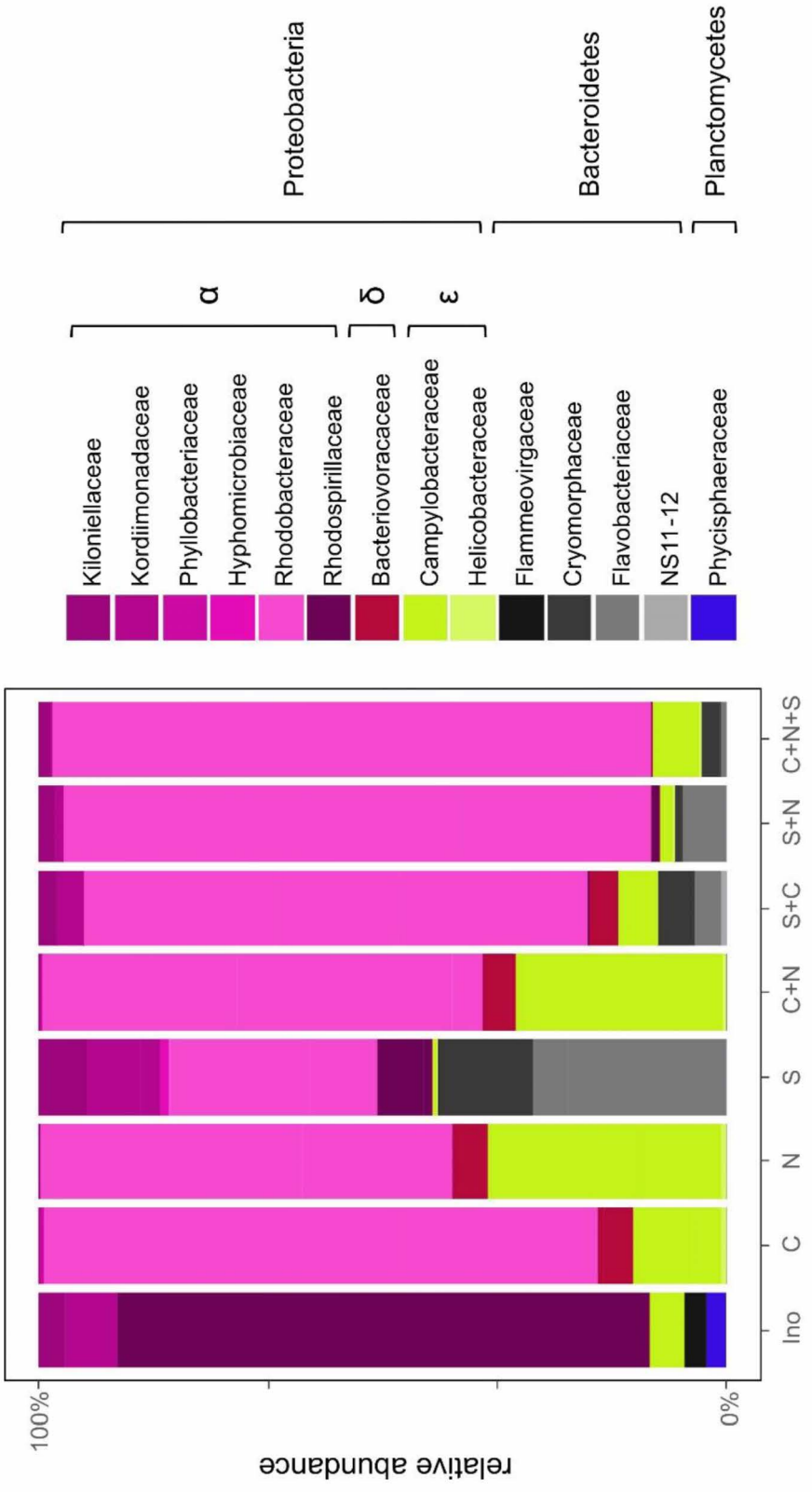

Figure 3: Bacterial community composition follows the diatom community. Relative bacterial abundances present in the original inoculum (Ino) or when grown with either *C. closterium* [C], *N. phyllepta* [N], S. robusta [S] or a combination of these diatoms. The different bacterial orders are depicted using different colours.

Multivariate analyses of the data (Supplementary Figure 6) showed a strong differentiation (PERMANOVA: p=0.006) between the bacterial communities associated with the different diatom combinations. The strongest difference was noticeable between the *S. robusta* monocultures and the cultures containing *C. closterium* and *N. phyllepta* (ANOVA-CCA: p=0.01), although there were some minor differences between the latter bacterial communities as well (ANOVA-CCA: p>0.05). The bacterial communities associated with *S. robusta* monocultures had a much higher diversity (H'= 2.01±0.15 based on the rarefied data) in comparison with the bacterial communities associated with the other diatoms (H'= 1.11±0.21 and H'= 0.87±0.09 for *C. closterium* and *N. phyllepta* monocultures, respectively).

Bacterial communities formed in the presence of *S. robusta* typically contained (amongst others) Alphaproteobacteria (*Thalassospira* sp., *Roseobacter sp.* and a *Kordiimonadaceae* sp.) and Bacteroidetes (*Mangrovimonas* sp. and *Owenweeksia* sp.). Especially the *Mangrovimonas* sp. tended to be highly abundant in *S. robusta* cultures (up to 42%) whilst being almost absent in *C. closterium* and *N. phyllepta* monocultures. In contrast, the bacterial communities of the latter monocultures were dominated (up to 80% of relative abundances) by an *Octadecabacter* species (Rhodobacteraceae Alphaproteobacteria) and to a lesser degree also by an unidentified *Arcobacter* sp. (Epsilonproteobacteria). Bacterial diversity in the diatom cocultures was not higher than in the monocultures (Supplementary Figure 7). Instead, an intermediate community structure was observed which was more similar to bacterial community present in the *N. phyllepta* and/or *C. closterium* monocultures. Finally, no linear relation (p>0.05) was found between bacterial diversity, as a whole or for different taxonomic groups, and diatom productivity.

Discussion

Our results show a positive relationship between benthic diatom diversity and productivity in simple experimental diatom communities. Although the biodiversity productivity relation has been shown to vary for diatoms at varying spatial scales and conditions (Baert et al., 2016; Blanco et al., 2012; Gessner et al.; Giller et al., 2004; Soininen, 2009), our results are in accordance with what was observed previously for marine benthic diatoms (Forster et al., 2006; Vanelslander et al., 2009). According to the biodiversity productivity relation, the positive effect of diversity on productivity could largely be attributed to complementarity effects (Loreau and Hector, 2001). This

indicated that the interspecific competition between diatoms was generally smaller in comparison to the intraspecific competition of the diatoms with bacteria and was likely the result of the improved usage of the available resources between the diatom species through cross-feeding and facilitation events (Bruno et al., 2003; Fridley, 2001). Although our experimental setup did not allow further analysis of EPS production and utilization, the presence of chemical cross-talk between diatoms, which include changes in the production, release and utilization of organic compounds, seems to be relatively common (Tuchman et al. 2006) and has been shown to stimulate the growth of cocultured diatoms (Paul et al., 2009; Vanelslander et al., 2009). For example, the spent medium derived from *N. phyllepta,* resulted in a mixotrophic switch that improved the growth rate of a specific *C. closterium* strain, by benefitting from the carbon released by *N. phyllepta* (Vanelslander et al., 2009). The capability of such a mixotrophic lifestyle, however, appears to be strain dependent (Mensens et al., 2015) and was not observed for the *C. closterium* strain used in this study (Audoor *unpublished data*). Regarding our results, a similar process however may be occurring in *S. robusta*, whose growth was positively affected by the presence of other diatom species and would require further testing.

Our experimental setup revealed the recruitment of distinct bacterial assemblages from a common bacterial inoculum by individual diatom species, which is in accordance with previous studies (Bagatini et al., 2014; Eigemann et al., 2013; Grossart et al., 2005; Jasti et al., 2005; Schafer et al., 2002; Sison-Mangus et al., 2014). Interestingly, despite an expected increase in substrate and habitat heterogeneity (Giller et al., 2004; Kassen et al., 2000), the combination of different diatom species did not result in a higher bacterial diversity. Neither could a relation be found between primary production and bacterial diversity, either as a whole or within certain taxonomic groups (Horner-Devine et al., 2003; Raes et al., 2018; Stein et al., 2014). Instead, the bacterial community composition of a mixture of diatom species reflected an intermediate combination of the bacteria that were present in the three separate diatom monocultures.

Although *S. robusta* and *N. phyllepta* are more closely related to one another, the bacterial community composition of *N. phyllepta* was more similar to that of *C. closterium*. *S. robusta* harboured a diverse bacterial community in comparison to the bacterial communities of *N. phyllepta* and *C. closterium* which were both dominated

by *Octadecabacter* sp., a member of the *Roseobacter* clade, and *Arcobacter* sp. The dominance of both bacteria over the other bacteria, coinciding with a negative impact on the growth rate of two out of the three diatom species, is a possible indicator of a negative and potentially allelopathic effect from these bacteria on other microorganisms (Mayali et al., 2008; Slightom and Buchan, 2009). A literature search revealed that not much is known about marine *Arcobacter* species (Collado and Figueras, 2011), but the genus does include several important mammalian pathogens (Ferreira et al., 2015). Several *Roseobacter* representatives, on the other hand, are known to reduce both algal and bacterial growth (Mayali et al., 2008; Seyedsayamdost et al., 2011; Sharifah and Eguchi, 2011), e.g. through the production of antibiotics (Brinkhoff et al., 2004; Bruhn et al., 2007; Ruiz-Ponte et al., 1999; Wagner-Dobler et al., 2004). Interestingly, the reduced manifestation of *Octadecabacter* and *Arcobacter* in the presence of *S. robusta*, whose growth rate was left unaffected in the presence of bacteria, could further suggest the ability of either *S. robusta* or its associated microbial community to suppress the growth of these bacteria or their potential antibiotic activity.

Although several studies have highlighted the species-specific effects of bacteria on diatom growth (e.g. Amin et al., 2012; Doiron et al., 2012; Grossart, 1999) and diatom community composition (D'Costa and Anil, 2011), our results indicate how these diatom-bacteria interactions may further impact the outcome of diatom-diatom competition and the subsequent effect on productivity. Indeed, few studies have investigated the impact of bacteria-host interactions on community functioning and whose results appear to differ (Gribben et al., 2017; Grime et al., 1987; Horner-Devine et al., 2003; Hortal et al., 2017; Hubbard et al., 1986; Kardol et al., 2007; Laforest-Lapointe et al., 2017; Pugnaire et al., 2004) and dependent on the environmental conditions (Callaway et al., 2004; Dittami et al., 2016; Marzinelli et al., 2018). In the absence of bacteria, experimental diatom communities were dominated by the species that showed the highest growth rate in monoculture, i.e. *C. closterium.* In mixed diatom cultures, the addition of bacteria negatively impacted the growth of both *C. closterium* and *N. phyllepta,* leading to the dominance of *S. robusta*. The presence of a bacterial community further altered the algal diversity functioning relationship, steepening the positive relationship found between diatom diversity and productivity as a result of an enhanced complementarity effect. Similar to what has been observed in plants

(Lekberg et al., 2018), the most competitive diatoms in our study experienced the most negative bacterial effects which in turn resulted in the bacteria indirectly promoting coexistence amongst the diatom species. Even if our simple experimental setup does not permit further extrapolation to field situations, our findings, using three naturally co-occurring diatom species, calls for a broader consideration of the role of benthic microbiota in shaping diatom community structure and function. Our findings fit well within the holobiont concept, which considers both hosts and their microbes as a single integrated unit (Zilber-Rosenberg and Rosenberg, 2008). Although it remains to be shown that coevolution between diatoms and bacteria is commonplace, as is often stated in this theory (Skillings, 2016), the strong interactions between host-diatoms and their associated bacteria have important implications for the overall diatom fitness and thus their established niche (Kopac and Klassen, 2016; Vandenkoornhuyse et al., 2015) in species-rich natural communities.

Data availability

The 16S rRNA raw sequence data were deposited in the NCBI Sequence Read Archive under the accession number PRJNA521472. The count data, growth rates and OTU table can be found on Github (https://github.com/willem-stock/Koedooder-et-al.-2019).

Acknowledgements

The research leading to results presented in this publication was carried out with infrastructure funded by EMBRC Belgium - FWO project GOH3817N. This research was supported under the BIO-Tide project, funded through the 2015-2016 BiodivERsA COFUND call for research proposals, with the national funders BelSPO, FWO, ANR and SNSF. WS was funded by the Fund for Scientific Research – Flanders (FWO-Flanders, Belgium). The BCCM/DCG diatom culture collection is acknowledged for providing us with the appropriate diatom strains for this study. The authors are very grateful for help of Sofie D'Hondt with the molecular work and Tine Verstraete for her aid with the experimental work. Bjorn Tytgat provided the mock community

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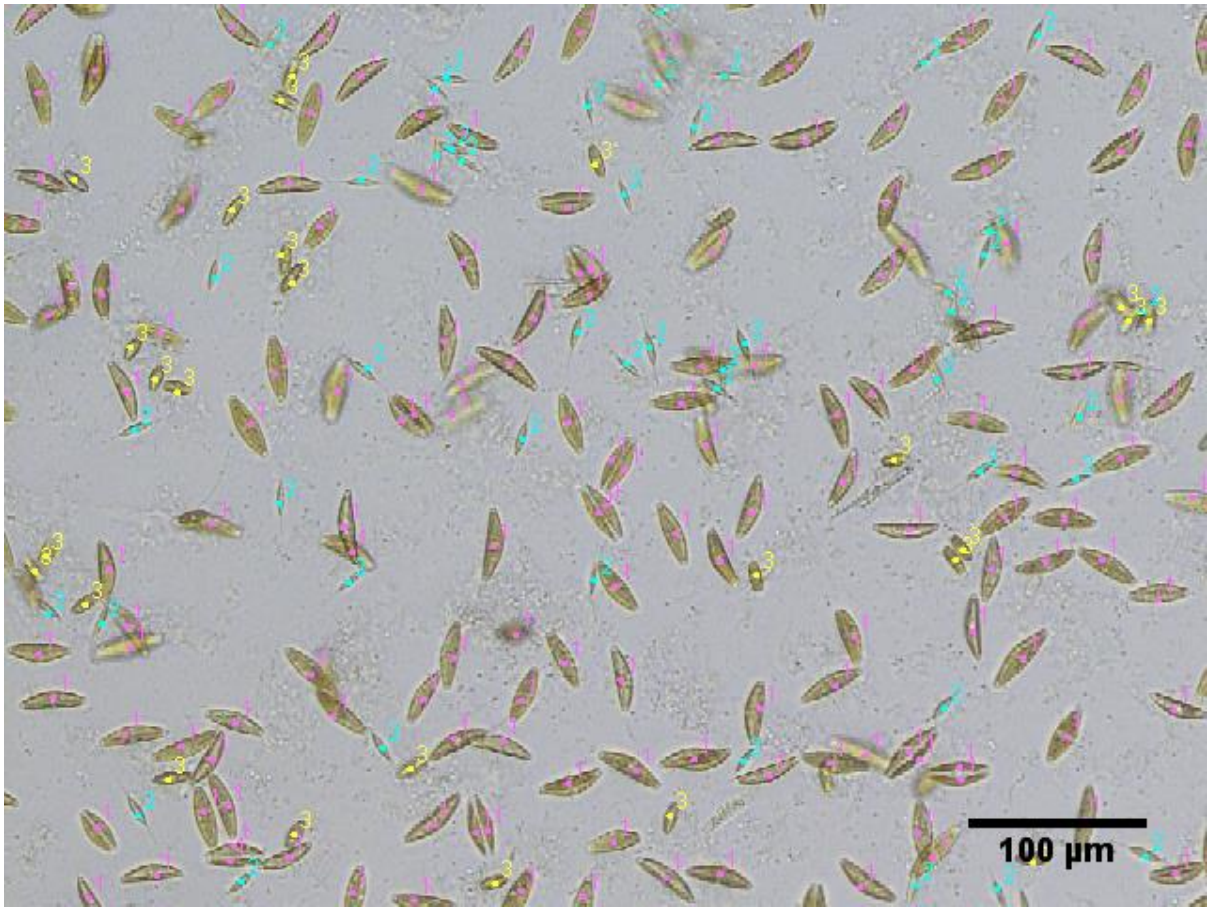
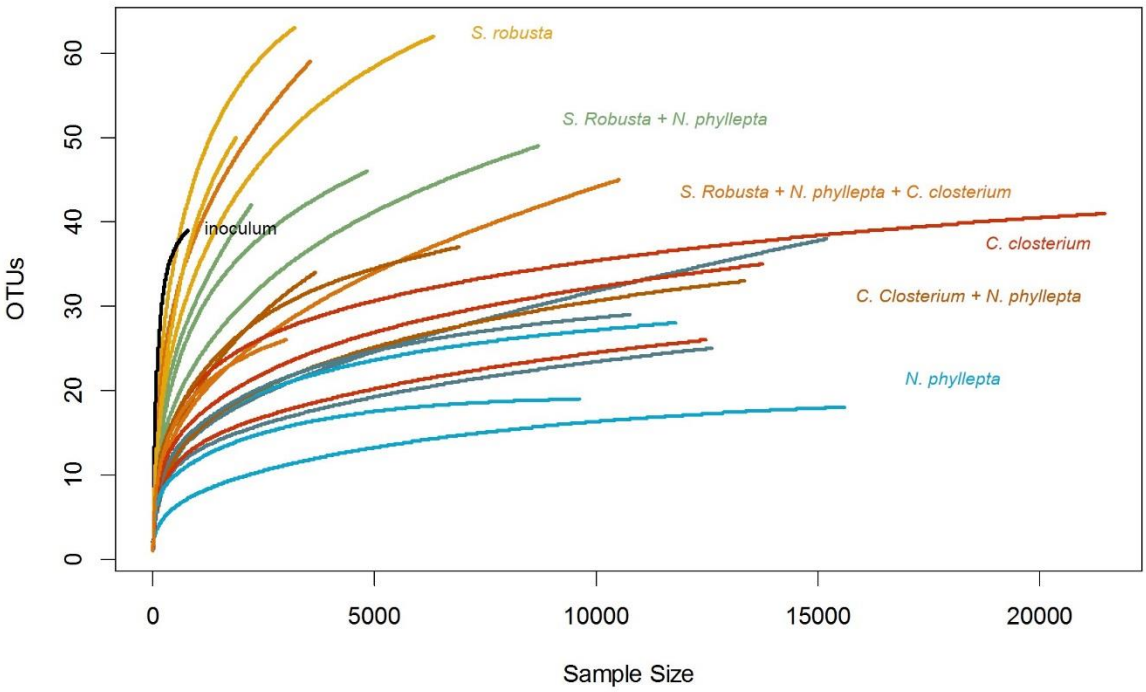
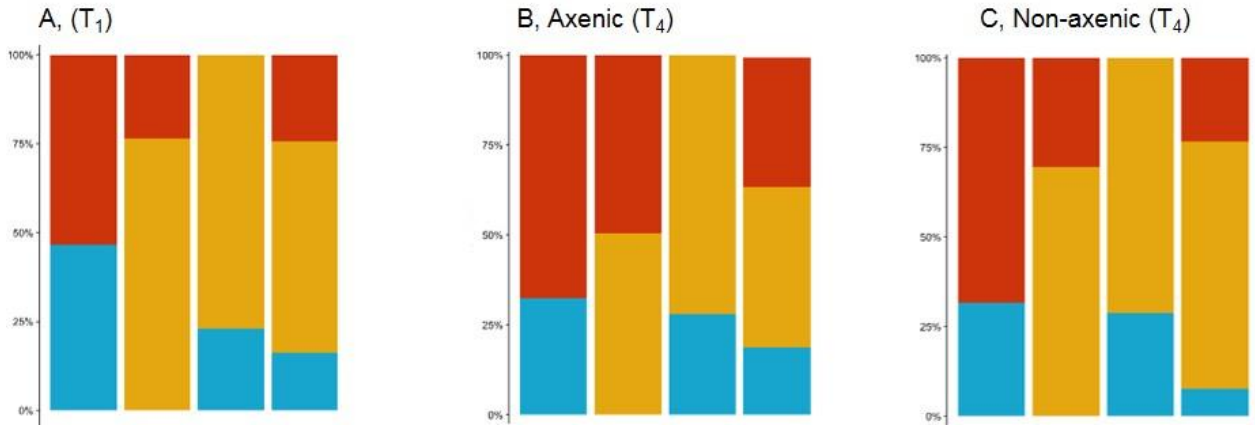
Supplementary material



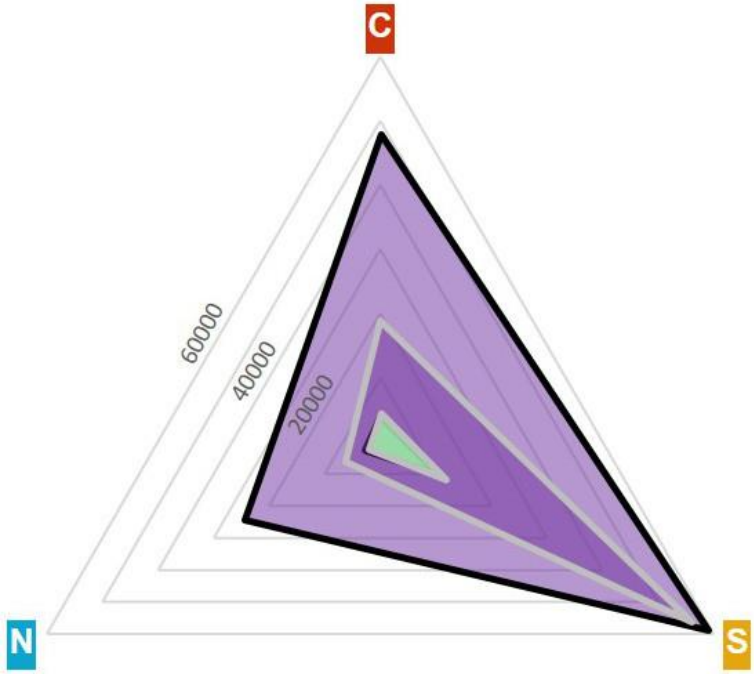
Supplementary Figure 1: Example of picture-based diatom cell counting. The image shows a mixed diatom culture inoculated with bacteria at day 3. The culture contains *S. robusta* (pink, nr. 1), *C. closterium* (cyan, nr. 2), *N. phyllepta* (yellow, nr 3). The image size is a fourth of the pictures taken for the cell counts.



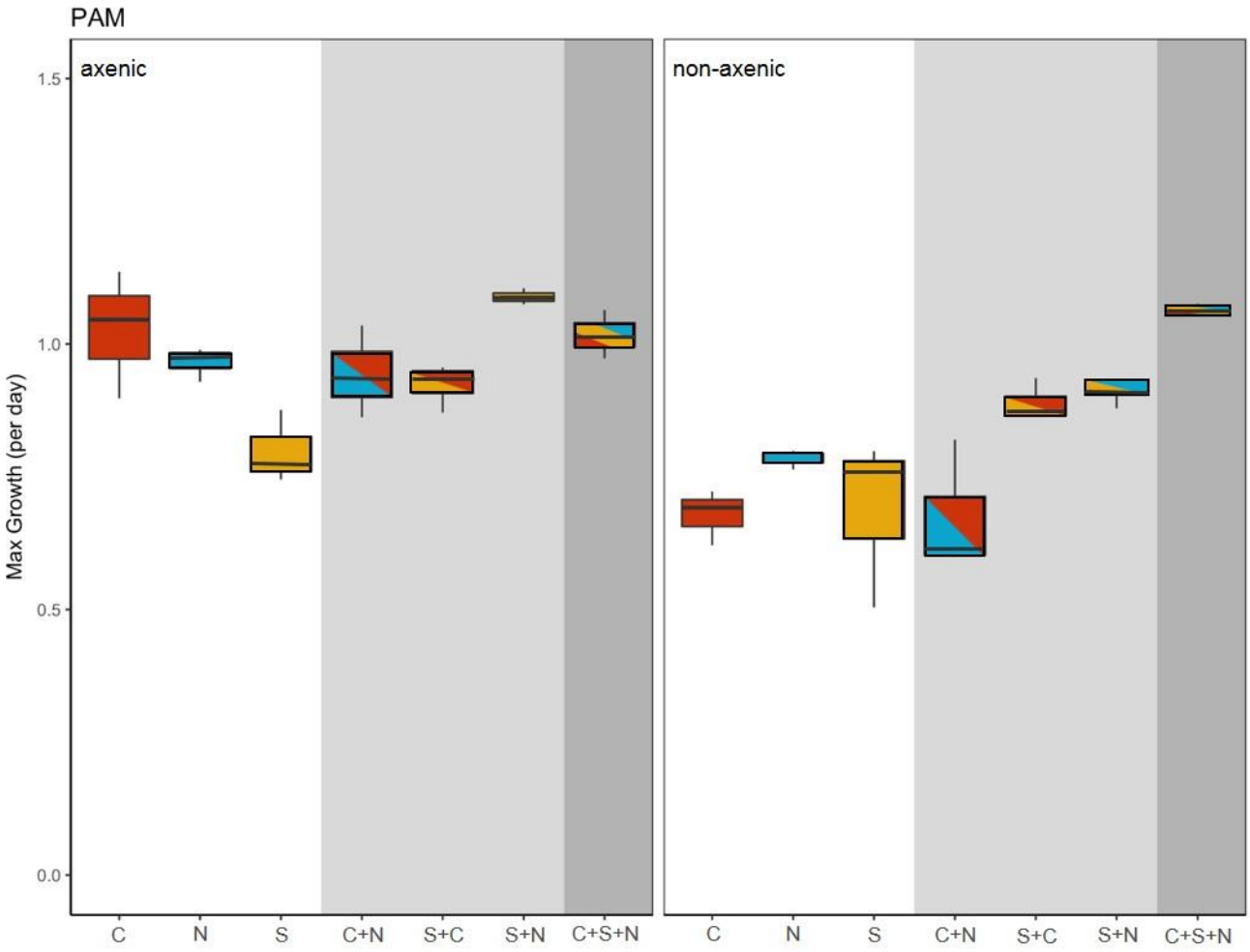
Supplementary Figure 2: Rarefaction curve for every sample (before removal of rare OTUs) is shown. Colors indicate the different treatments as displayed.



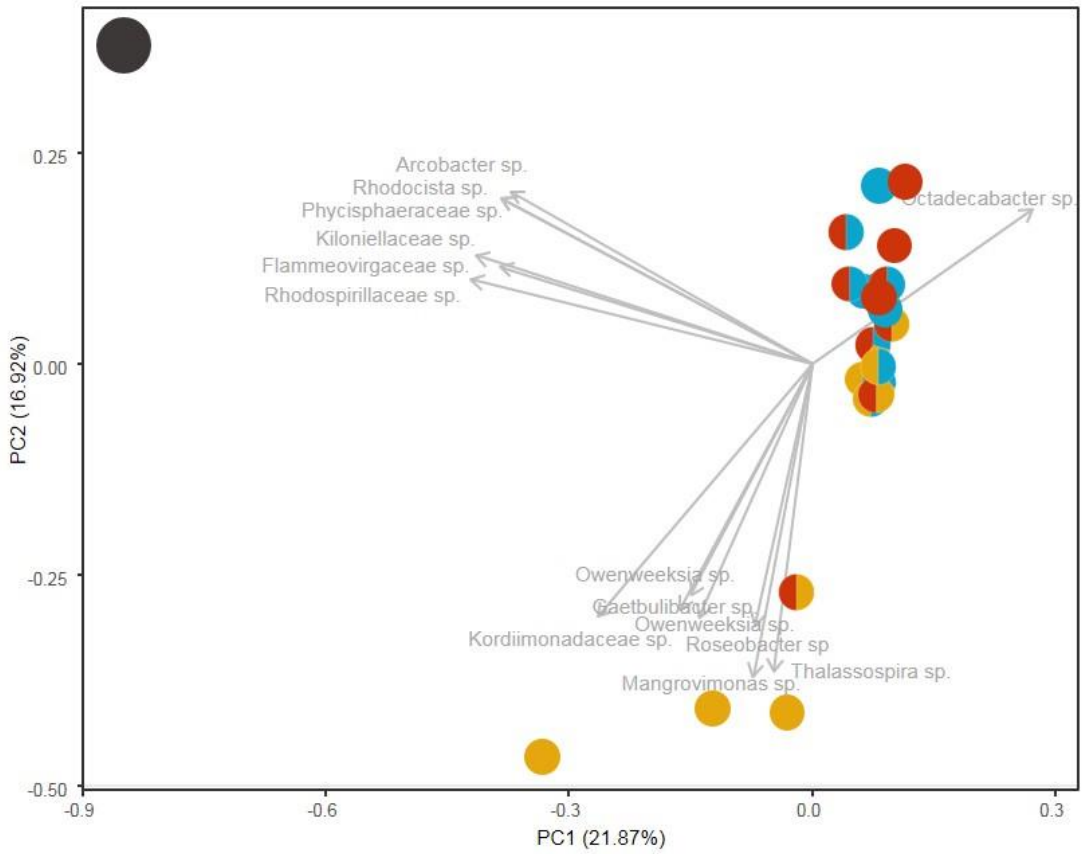
Supplementary Figure 3: The presence of bacteria causes a shift in the relative proportions of diatoms. The relative proportion of *C. closterium (red), S. robusta (yellow)* and *N. phyllepta (blue)* grown in pairs or altogether expressed as the number of cells per species to the total number of cells in the well. (Left = T_0, Centre = T_4 in axenic conditions, Right = T_4 in non-axenic conditions. Briefly, in axenic conditions an increase in the proportion of *C. closterium* is observed when grown in the presence of *S. robusta*, while this observation was not observed in the presence of bacteria. Instead, *S. robusta* had an increased overall proportion in comparison to *C. closterium*.



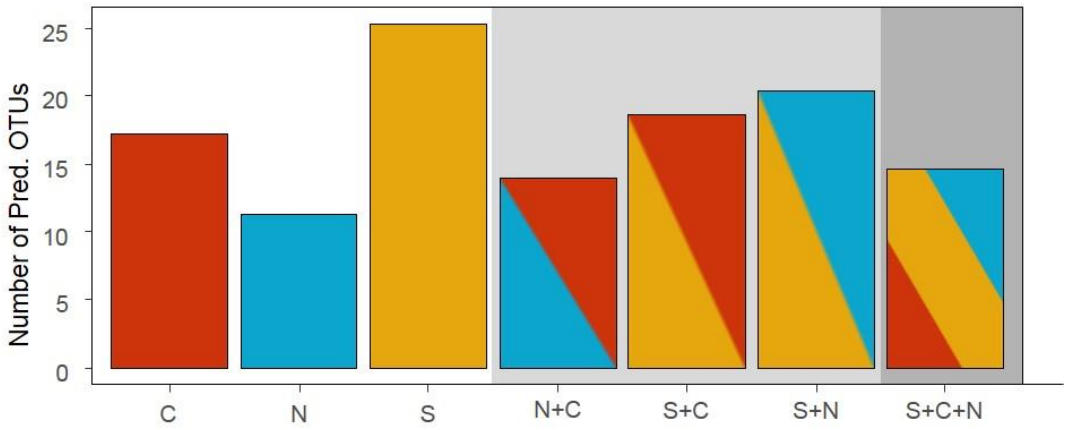
Supplementary Figure 4: The change in the total biovolume (µm³) for each of the three diatom species [*C. closterium* (red), *N. phyllepta* (blue) and *S. robusta* (yellow)] one day after inoculation (green area) and four days after inoculation (purple area). The grey line indicates the biovolumes under non-axenic conditions while the black line specifies those under axenic conditions.



Supplementary Figure 5: PAM measurements depicting the total fluorescence as a proxy for the total maximum growth rates for different combinations of *C. closterium* ([C], red), *N. phyllepta* ([N], blue) and *S. robusta* ([S], yellow) in the presence (non-axenic) or absence of bacteria (axenic). Combinations of multiple diatoms species together have the appropriate colours combined. The white, grey and dark grey background respectively show diatoms grown in monocultures, diatom pairs or the three diatom-species combined.



Supplementary Figure 6: PCA on the relative abundance of the bacterial community from the original inoculum (black circle) and when grown in the presence of different combinations of *C. closterium* (red), *N. phyllepta* (blue) and *S. robusta* (yellow). Samples with more diatoms present have circles with the appropriate colours combined. Grey arrows indicate the vectors of the bacterial OTUs with a cumulative loading larger than 30% on the first two axes. The bacterial genus or family is indicated depending on the closest hit. The proportion of the variance explained by each axis is indicated next to that axis.



Supplementary Figure 7: Diversity levels of bacteria in the presence of different diatom combinations of *C. closterium* (C, red), *N. phyllepta* (N, blue) and *S. robusta* (S, yellow). Bacterial community diversity was calculated as the average number of predicted OTUs after rarefication of the samples to 781 reads.

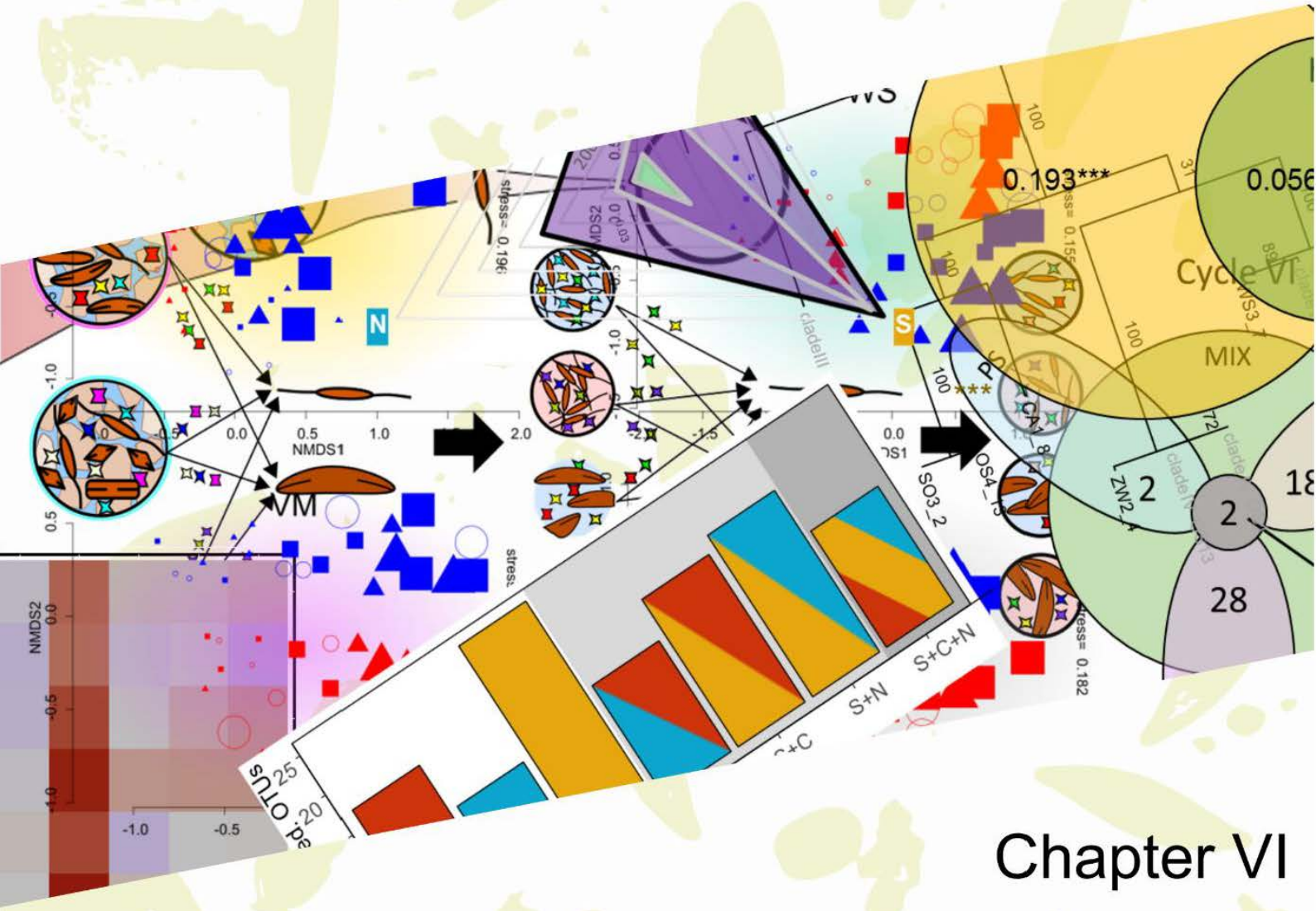
Supplementary Table 1: Information on the 3 different diatom strains used within our experimental setup. More information can be found at the Belgian Co-Ordinated Collections of Micro-organisms website using the diatom accession number (http://bccm.belspo.be/catalogues)

Accession #	Diatom species	Strain	Isolation date	Isolation location
DCG 0623	*Cylindrotheca closterium*	WS3_7	20 May, 2014	Paulina Schor, NL (51° 21' 1.9188'' N; 3° 43' 34.4388'' E)
DCG 0105	*Seminavis robusta*	85A	8 August, 2006	Progeny of strains from Veerse Meer, NL (51°32'36" N; 3°48'15" E)
DCG 0476	*Navicula phyllepta*	PBA12	10 January, 2013	Paulina Schor, NL (51°21' N; 3°43' E)

Supplementary Table 2: Statistical analysis of the total maximum growth rates derived from F_0 PAM values of 24-plate wells harbouring different combinations of S. robusta [S], C. closterium [C], and N. phyllepta [N]. A two-way ANOVA was conducted testing the differences between the total growth rate of the diatom combinations (single, paired or all together) and the presence or absence of bacteria. A posthoc (Tukey Test) was further conducted on each of the different diatom combinations. An asterisk marks results of significant value where * < 0.05, ** <0.01, and *** < 0.001.

F_0 PAM					
Two-way ANOVA	**Factor**	**DF**	**F value**	**p-value**	**significance**
	Diatom combination	6	11.558	$1.7x10^{-6}$	*******
	bacteria - axenic	1	43.346	$3.86x10^{-7}$	***
	interaction	6	4.844	$1.67x10^{-3}$	**
		28			
Post hoc	**Diatom combination [axenic – bacteria]**	**Difference**		**p-value**	**significance**
SINGLE					
	[c]	-0.348		$3.0x10^{-4}$	***
	[s]	-0.11		0.858	
	[n]	-0.181		0.213	
PAIRED					
	[s+n]	-0.175		0.26	
	[s+c]	-0.0343		0.10	
	[n+c]	-0.269		0.0087	**
TRIPLE					
	[s+c+n]	0.049		0.999	

Chapter VI: General discussion and perspectives



Chapter VI

General discussion and perspectives

Throughout this thesis, we have manipulated different aspects of the interactions between marine benthic diatoms and heterotrophic bacteria (Figure 1) to get a better understanding of the underlying processes shaping these associations. By characterising the bacterial communities associated with benthic diatoms and the nature of the interactions between bacteria and these diatoms, we assessed which bacteria were relevant, how specific these associations were, if this specificity was beneficial to the diatom and if it could impact the structure of a benthic diatom community.

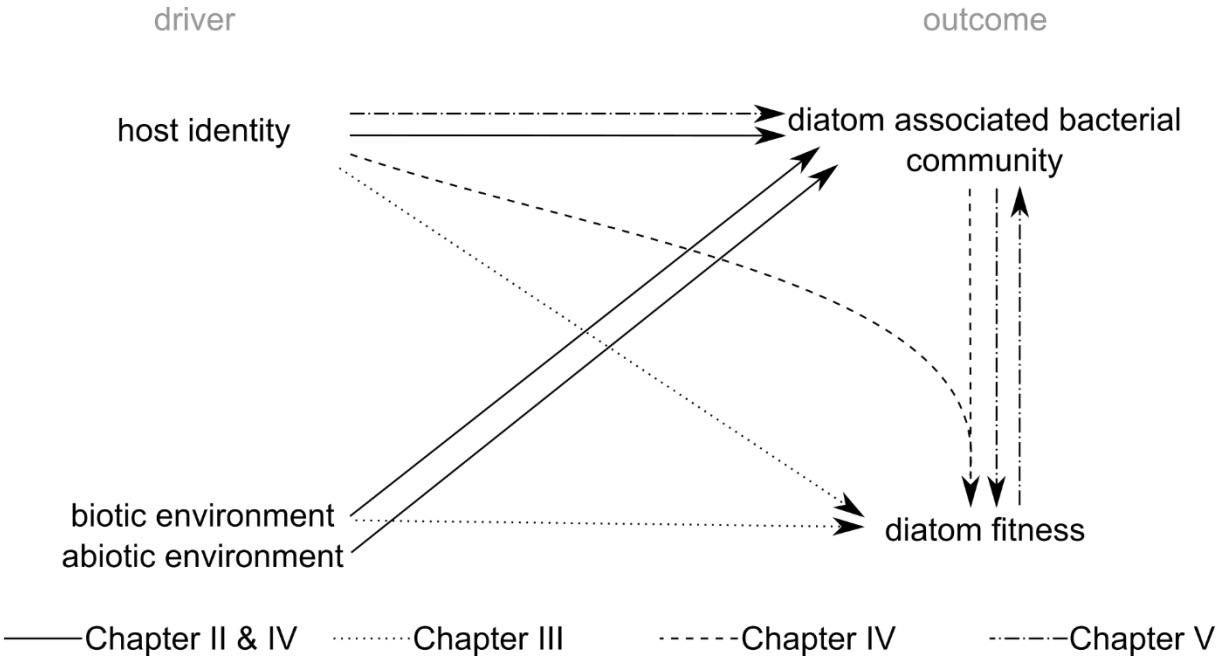


Figure 1: Schematic representation of the links made in this thesis. By manipulating the diatom host(s) present (*host identity*), the *abiotic environment* (e.g. nutrient concentrations), the bacteria present (*biotic environment*) or a combination thereof, we identified the effects of the host and the environment on the structure and composition of the *diatom associated bacterial community* and the growth of the diatom (*diatom fitness*). The arrows indicate the relations that were tested and the line style indicates in which chapter they were assessed.

Throughout the different studies in this thesis, we have observed reoccurring associations between benthic diatoms and heterotrophic bacteria, particularly when considering the higher taxonomic ranks (order-family). At a finer taxonomic level (genera-species and OTUs), both the associations and the nature of the interactions proved to be highly specific. In what follows, we discuss our findings and interpret these in an eco-evolutionary context. We consider the *in situ* relevance of these findings and, finally, we provide future recommendations for benthic diatom-bacteria research.

Diatom-associated bacteria: composition and diversity

Comparing the bacterial communities in the diatom cultures to the natural samples they were originating from, we observed a remarkable yet consistent decrease of diversity in combination with a persistent enrichment of selected bacterial groups in the presence of diatoms.

The Rhodobacteraceae (Alphaproteobacteria; Rhodobacteriales) were in all studies well represented, both as isolates (chapter III) and in the 16S rDNA amplicon sequencing datasets (Chapters II, IV, V; Figure 1). More specifically, most of the strains and OTUs belonged to the *Roseobacter* group, including representatives of the genera *Celeribacter* (Chapter II, III, IV), *Loktanella* (Chapter II, III), *Octadecabacter* (Chapter II, V), *Phaeobacter* (Chapter II), *Roseobacter* (Chapter V) and *Sulfitobacter* (Chapter II, III, IV). This most likely paraphyletic and highly diverse group (Simon et al. 2017) is commonly found to be associated with diatoms (Amin et al. 2012; Behringer et al. 2018; Grossart et al. 2005). Due to their commonness and important role in various biogeochemical cycles, this group has been studied in detail (Daniel et al. 2018 and references therein), yet their ecological significance and the evolutionary processes shaping their genomes of this group is still incompletely known (Daniel et al. 2018). Many studies have reported extensive metabolic interchanges between *Roseobacter* group members and microalgae (Amin et al. 2015; Barak-Gavish et al. 2018; Geng & Belas 2010) and similarly, this group has been suggested to exert significant control over phytoplankton biomass and community structure in the oceans (Mayali et al. 2008). Members from this group hold the potential to greatly enhance (Geng & Belas 2010) or inhibit (Sonnenschein et al. 2018) algal growth. Moreover, these bacteria have developed mechanisms by which they can regulate their pathogenicity depending on the physiological status of their host (Barak-Gavish et al. 2018; Seyedsayamdost et al. 2011). In the present study, none of the isolates from this group strongly impacted the growth of *Cylindrotheca closterium* (Chapter III), but an *Octadecabacter* strain did seem to be involved in growth inhibition of both *C. closterium* and *Navicula phyllepta* (Chapter V). The growth recovery of the diatoms in Chapter IV was accompanied by an increase in *Roseobacter* group members, suggesting a positive influence of these bacteria on diatom growth. Our observations therefore do suggest that representatives of the *Roseobacter* group can have a substantial impact on the dynamics of diatom-bacteria consortia. It is worth pointing out however that the *Roseobacter* group was

also abundant in the diatom-free controls in chapter IV. As many members from this group are well-adapted to colonize a wide range of substrates including plastics (Osborn & Stojkovic 2014) or other marine organisms such as echinoderms (Morrow et al. 2018), the generally high abundance of this group in the HTP sequencing data from Chapters II, IV and V, does not necessarily imply reciprocal interactions between all detected *Roseobacter* group members and their diatom hosts. Maybe some of these bacteria might merely experience the diatoms (or the confinements of the well) as a suitable substrate for attachment.

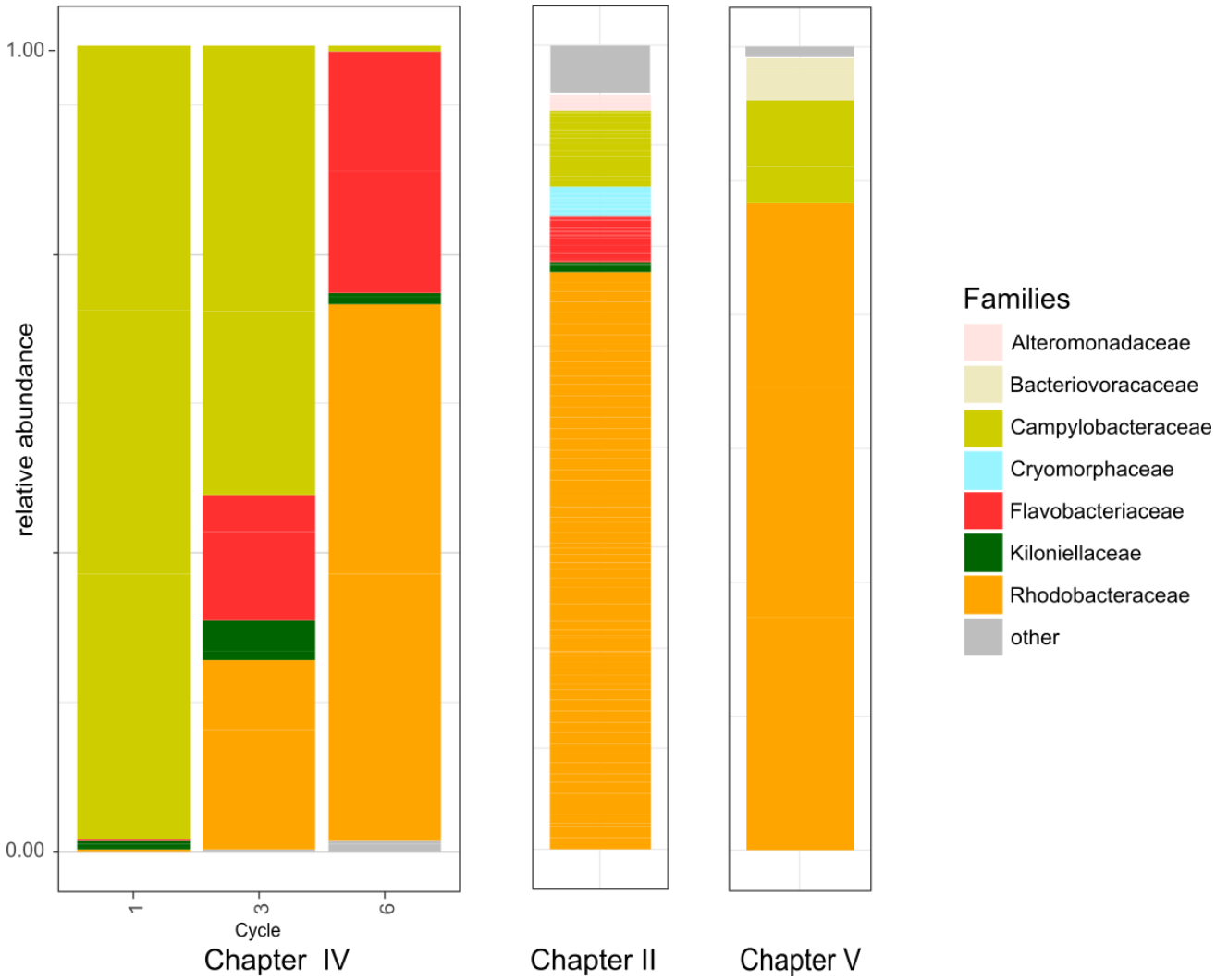


Figure 2: relative abundance of the bacterial families associated with *C.closterium* cultures across different studies. The average relative abundance of the most common bacterial families in, from left to right, the WS inoculated *C. closterium* cultures over the first, third and sixth cycle (Chapter IV), all *C. closterium* cultures from Chapter II and the *C. closterium* cultures from Chapter V.

Two other proteobacterial genera are worth noting: *Marinobacter* (Gammaproteobacteria; Alteromonadales) and *Arcobacter* (Epsilonproteobacteria;

Campylobacterales). Several *Marinobacter* strains were isolated from *Seminavis robusta* cultures (Chapter II), including two that strongly inhibited the growth of *C. closterium*. Remarkably, no *Marinobacter* spp. were detected in the HTP 16S rDNA sequencing datasets from the diatom cultures or the environmental samples. *Marinobacter* spp. are not uncommon in marine sediments (Handley & Lloyd 2013) or in association with microalgae (Amin et al. 2009, 2015; Baker et al. 2016; Crenn et al. 2018). The lack of *Marinobacter* spp. in the sequencing data might therefore be an artefact (see below) or, alternatively, this genus may effectively be rare in a benthic setting and in benthic diatom cultures. *Marinobacter* was previously reported to be less common in some planktonic diatom cultures than in dinoflagellate and coccolithophorid cultures (Amin et al. 2009 but see Baker et al. 2016). Due to the strong diatom growth inhibition caused by half of the *Marinobacter* isolates (Chapter III) and previously reported pronounced interactions with planktonic diatoms (Amin et al. 2009; Gärdes et al. 2012; Sonnenschein et al. 2011; Wang et al. 2014) the potential presence and importance of *Marinobacter* in a benthic algal context deserves further study.

The second proteobacterial genus, *Arcobacter*, was highly abundant in the first growth cycles of axenic diatom cultures that had been inoculated with natural marine bacterial communities (Chapter IV), relatively well-represented in the microbiomes of freshly isolated *C. closterium* strains (Chapter II) and was the second most abundant genus in Chapter V. Its presence coincided with reduced diatom growth (Chapter IV-V). Most of what is known to date about this genus is related to its function as a mammalian pathogen (Collado & Figueras 2011; Salas-Massó et al. 2016). It has been reported from macroalgae (Kim et al. 2010; Sweet et al. 2013) and intertidal sediments in association with *Spartina alterniflora* roots (McClung et al. 1983). Apart from being reported to co-vary with diatom presence during the incubation of marine sediment cores (Broman et al. 2019), no specific interactions have yet been reported between diatoms and *Arcobacter* species. *Arcobacter* might, together with the *Marinobacter*, be a carbon opportunist (Evans et al. 2018). This less acknowledged genus therefore also requires further study to confirm the potentially growth-inhibiting effect observed in our studies. Particularly the differential response between *S. robusta* and *C. closterium* when exposed to this genus, as reported in chapter V, could be interesting to investigate as an example of species-specific interactions.

Although generally less abundant than Rhodobacteraceae, Flavobacteriaceae were also an important group in all our studies. Flavobacteriaceae were reasonably diverse in most HTP 16S rDNA sequencing datasets (Chapter II, V, IV) and representatives of three different genera from this family were isolated, two of which proved to be strongly growth-inhibiting (Chapter III). These bacteria live generally attached to particles (Fernández-Gómez et al. 2013) or diatoms as was shown by Grossart et al. (2005) who identified more bacteria from this group in the fraction attached to diatoms than in the suspended fraction. They are recognized for their capacity to degrade polymers (Fernández-Gómez et al. 2013) and play a significant role in the degradation of organic matter produced by microalgae (Abel & Bowman 2005). The abundance of this group differed between *C. closterium* and *S. robusta* in chapter IV and V. There were more Flavobacteriaceae associated with *C. closterium* in chapter IV, while the opposite was true in chapter V: Flavobacteriaceae were absent in the *C. closterium* cultures whist a *Mangrovimonas* sp. made up approximately one third of the *S. robusta* associated bacterial communities. These differences can probably be attributed to differences in the bacterial source communities and how they were treated (see below). Nonetheless, at least on two occasions did the Flavobacteriaceae strongly contribute the differences in the bacterial community composition between diatom species.

Specificity

The diatom-associated bacterial communities observed in this thesis, largely made up of the previously discussed Rhodobacteraceae, Flavobacteriaceae, but also including Campylobacteraceae (Epsilonproteobacteria, including *Arcobacter*), Kiloniellaceae (Alphaproteobacteria) and several other families (Figure 2), tended to be species- (Chapter IV –V) or even strain-specifically (Chapter II) structured. Host-specificity has repeatedly been shown for diatom-associated bacteria (Behringer et al. 2018; Crenn et al. 2018; Grossart et al. 2005; Schäfer et al. 2002), even when comparing closely related species or strains (Sison-Mangus et al., 2014). It has however also been questioned (Kaczmarska et al. 2005; Sapp et al. 2007a). In addition the host-specific effects, differences between diatom-associated bacterial communities could also be related to the environment (Chapter II, IV). The influence of the environment was, arguably, even more pronounced than that of the host (Chapter II). Notably, the influence of the environment could result from the environmental conditions (e.g. differences in nutrient concentrations) or differences between regional bacterial source

pools. Conflicting statements have been made about the influence of the environment. Whilst some studies reported a very limited influence of the environment on the algal microbiome (Eigemann et al. 2013; Sapp et al. 2007), others found that environment did have a pronounced effect (Grossart 1999; Baker et al. 2016), perhaps even more so than the potential differences between species (Ajani et al. 2018).

Comparisons between these studies and ours are hampered by methodological differences. Some have analysed *in situ* bacterial communities associated with diatoms (Baker & Kemp 2016; Crenn et al. 2018), but most have compared the bacteria present in diatom cultures. For a handful of studies, these cultures were recently initiated from a single cell as was done in chapter II (Ajani et al. 2018; Behringer et al. 2018; Guannel et al. 2011; Sison-Mangus et al. 2014). From these, Behringer et al. (2018) reported species-specific microbiomes based on persistent differences between 3-4 strains of *Nitzschia longissimi* and one *Asterionellopsis glacialis* respectively. Sapp et al. (2007b) also reported differences between several common phytoplankton species. Guannel et al. (2011) and Sison-Mangus et al. (2014) noted marked differences between the bacterial communities associated with different species within the genus *Pseudo-nitzschia*. The authors argued that these differences might be attributed to differences in domoic acid production between strains or species. By extension, Jasti et al. (2005) compared bacterial communities from recently isolated *Alexandrium* strains, a toxic dinoflagellate genus, with several other phytoplankton species (including several Coscinodiscophyceae). More bacteria were shared between the *Alexandrium* strains than between *Alexandrium* and the other genera. In contrast, Ajani et al. (2018), who compared closely related *Leptocylindrus* species, reported no effect of strain or species. The same study does report a strong temporal and spatial variability on the diatom microbiomes. Taken together, most studies report differences in the composition of associated microbiota between microalgal species, when species sufficiently differ for one another, either by being phylogenetically distinct or by differing in toxin production. By linking the phylogenetic relation of the hosts with the differences in their microbiomes (Chapter II), we could infer host specificity at a finer scale than the studies listed above. This effect was nonetheless small and would otherwise have been missed, for example in the case in Ajani et al. (2018) study. The strong environmental influence reported in the latter study is in line with our findings.

In chapters IV & V, we found microbiomes to be species-specific, after axenic algal cultures had received a bacterial inoculum. This allows to control for differences in bacterial inoculum, which was not possible in the above-mentioned studies and in chapter II. Only one study used a similar setup to compare the microbiomes between diatom species (Grossart et al. 2005), and this study also reported species-specific microbiomes. Notably, a study with the same design reported different microbiomes between cyanobacteria and the diatom *Aulacoseira granulata* (Bagatini et al. 2014). Chapter IV also showed a large effect of the inorganic and organic resource supply and type on the algal microbiomes, which corroborates previous findings (Grossart et al. 1999; Baker et al. 2016). Notably Eigemann et al. (2013) did not find a strong influence of to the different environmental conditions to which the algae were exposed, but that might have been because the algal cultures were not axenic at the beginning of the experiment.

Community assembly in diatom-associated bacterial assemblages

By identifying the underlying processes responsible for shaping the algal microbiomes, we hoped to explain the observed patterns in the diatom-associated bacterial communities. To this end, we compared diatom-associated bacterial communities to randomized communities (Chapter II), related the effect of bacterial strains to their origin (same or different host; Chapter III) and challenged diatoms with bacterial communities that differed in their relation to the host (sympatric or allopatric; Chapter IV).

Ecological selection

Ecological selection, the most explored process involved in community assembly (Vellend 2010), was readily identified in our different studies (Chapters II-V). Selection was driven by both abiotic factors (environmental conditions) and biotic interactions (with other bacteria and with the diatom hosts; Nemergut et al. 2013). Abiotic selection was apparent in chapter IV where diatom-associated bacterial communities differed substantially between the two media used. In chapter II, the influence of the environmental variables (such as benthic or planktonic and the nutrient concentrations) was large (explaining over ten percent of the variation in the *C. closterium* microbiomes). However, due to the design of the study, we were not able to disentangle abiotic selection from dispersal or priority effects. Although not reported in this thesis, priority effects were observed during preliminary experiments, thereby

confirming the relevance of such effects in this context. Biotic selection was apparent in chapters II-V and occurred at different scales: within and between hosts. This resulted in a seeming contradictory selective enrichment of bacterial taxa amongst algal microbiomes but also substantial differences between microbiomes associated with phylogenetically different hosts. In all our studies, selective enrichment consistently resulted in a diatom core microbiome of *Roseobacter* group members. Many culture-based studies concluded the same (e.g. Ajani et al. 2018; Behringer et al. 2018), and this was also observed in other microalgae (Green et al. 2004; Hattenrath-Lehmann & Gobler 2017; but see Lupette et al. 2016). The high prevalence of this group as a whole is therefore most likely the result of the selective enrichment in microalgal cultures, independent of the identity of the algal host itself.

In chapter II, we could not differentiate between host-driven selective enrichment and artefacts introduced by the isolation procedure i.e. random bacterial contaminants picked up during the single cell isolation of diatoms from natural samples, followed by two growth cycles in well plates. This problem was eliminated in Chapter IV by including wells without diatoms. As the bacterial communities in those wells were remarkably similar to the wells with diatoms, the effect of the isolation procedure in chapter II was likely substantial. We remark that Crenn et al. (2018) also reported a high prevalence of *Roseobacter* group members associated with individual diatom cells isolated from natural environments. We therefore suspect the prevalence of the *Roseobacter* group to be truly the result of the selective enrichment by the algal host and not an artefact from the culturing procedure.

The differences between the diatom hosts resulted in the host-specific microbiomes (Chapters II-V) as described above. The extent of host selection was difficult to quantify as evidenced in chapter II where the variation partitioning did not fully uncover host selection effects that were identified by the randomisations. As reported for macroalgae (Burke et al. 2011) we also found indications that the selective enrichment by the host should also be considered in a functional context (Chapter II) and, although not further pursued in this thesis, apart from the Biolog Ecoplates in chapter IV, this is a promising avenue to asses host selection in future research.

In addition to selection by the host on the bacteria, interactions between bacteria themselves resulted in biotic selection (Nemergut et al. 2013). The results of chapter II suggested bacteria to affect each other positively (positive co-occurrence of bacteria

as compared to the randomisations), but later experiments mostly showed negative effects between bacteria: the growth-inhibiting *Marinobacter* was outcompeted by other marine bacteria in chapter III and almost all plant soil bacteria rapidly disappeared when mixed with marine bacteria. The latter results show that bacterial interactions might inhibit the establishment of less well-adapted bacteria.

Drift

Neutral processes, referred to as ecological drift in Vellend (2010), result from stochasticity in the growth dynamics of bacteria. As a result of random variation in population sizes, all but one species will eventually go extinct if no new species are introduced (Hubbell 2001). Once our diatom cultures were initiated (be it from single cell isolates or by inoculation of axenic diatoms), no new bacteria were introduced, and we can thus expect (in the absence of contamination or rapid speciation) a decrease in bacterial diversity due to drift. We tested for such a decrease in chapter IV but could not detect one. Also other studies do not report such a decrease of bacterial diversity in diatom cultures (Behringer et al. 2018; Schäfer et al. 2002). In contrast, these studies report relatively stable microbiomes, which we would not expect under drift.

In the absence of selection, the establishment success of bacteria should be proportional to their initial abundances. As selective enrichment was repeatedly observed (Chapter II, IV & V, see above) whereby certain taxa (e.g. Rhodobacteraceae & Campylobacteraceae) were consistently more abundant in the cultures than they were in the *in situ* samples, drift does not seem to be apparent. However, at the finest taxonomic levels (OTUs), the outcomes of the different experiments were less consistent. In the *C. closterium* cultures in chapter II, we reported a prevalence of bacteria which could be directly related to the abundance of those bacteria in the *in situ* samples, suggesting that there is a lasting imprint of the *in situ* bacterial community on the diatom culture associated one (Lankau & Mackie 2012). Furthermore, the dominant bacterial families were represented by different OTUs in the different algal microbiomes. For instance, in chapter II, many differences in the presence of specific genera from within the *Roseobacter* group were observed between the different *C. closterium* microbiomes. In chapter IV, *Celeribacter* and *Phaeobacter* were the most abundant *Roseobacter* group genera in the diatom cultures started from the WS inoculum whilst in the diatom cultures from chapter V, inoculated with a bacterial community from that same location, *Octadecabacter* and *Roseobacter* were the most

prominent *Roseobacter* group members. Although there were minor differences between these studies in terms of cultivation conditions (medium & light), the different outcomes could also be the result from stochastically amplified differences between bacterial inocula (see below). As closely related bacteria are likely more functionally similar (Martiny et al., 2013; Morrissey et al. 2016), selection effects are probably less important at the finer taxonomic levels, making way for noticeable drift effects. Based on our findings, we thus report a minor but significant role of ecological drift in the assembly process, which is more or less in accordance with what has thus far been reported for many other horizontally transmitted host associated bacterial communities such as gut microbiomes (Burns et al. 2016; Li & Ma 2016; Lankau & Mackie 2012)

We do however also remark that in many cases the perceived stochasticity can be due to the experimental design of the studies. The sampling effect whereby a restricted set of bacteria was introduced in the diatom cultures (in chapter II, during the isolation procedure only the bacteria on or in the immediate vicinity of the diatom were transferred; in chapters IV & V, a bacterial inoculum was added only once, at the beginning of the experiment) reduced the potential for competitively superior bacteria to be present in the later stages of the assembly process. Since no new bacteria were introduced later on, competitive replacement between bacteria could have been less pronounced than under *in situ* conditions.

We also note that, due to these same restrictions imposed by our experimental design, the influence of bacterial movement towards or away from the diatom could not be assessed. Active dispersal is nonetheless expected to play an important role in the dynamics between bacteria and diatoms (Amin et al. 2012; Raina et al. 2019). Motility and chemotaxis may be a way for bacteria to maintain spatial proximity to diatom cells, providing them with a fitness advantage (Seymour et al. 2017). The role of active bacterial motility towards diatoms in a planktonic setting has been studied quite extensively (Grossart et al. 2001; Smriga et al. 2016; Sonnenschein et al. 2010; Sule & Belas 2013). The motility of bacteria living in the sediment is similar to that of planktonic bacteria (Fenchel 2008) and is expected to be particularly prevalent in the top layers of the sediment, where small-scale heterogeneity promotes such behaviour (Petro et al. 2017). The contribution of bacterial motility in the assembly processes of bacterial communities around benthic diatoms still remains to be determined.

Coevolution and coadaptation

In addition to ecological processes such as selection and drift, evolutionary dynamics between bacteria and diatoms can also play a role in the assembly process of diatom-associated bacterial communities, and *vice versa*. Several studies have shown that bacteria are characterized by far-reaching adaptions towards their host (e.g. chemotaxis towards their host (Sonnenschein et al. 2012); interference with the cell cycle of the host (Van Tol et al. 2016)) and *vice versa* (Amin et al. 2015; Durham et al. 2017), but they do not relate these adaptations to the presence or absence of bacteria in diatom microbiomes. Studies focused on characterising the bacterial communities associated with diatoms (e.g. Schäfer et al. 2002) generally do not deal with the underlying evolutionary processes, which might lie at the basis of differences between bacterial communities. In the present study we therefore compared evolutionary relationships between different hosts with the structure of their bacterial communities (Chapter II) or their physiological response (i.c. growth rate) towards bacteria (Chapter III & IV).

In chapter II we reported a minor but significant effect of the host phylogeny on the structure of the bacterial community. This 'phylosymbiotic' signal (O'Brien et al. 2019) might reflect a shared evolutionary history between different representatives within the *C. closterium* species complex and associated bacteria (*sensu stricto* as in Herrera (1985)). However, the signal may also be the result of more closely related hosts also having more similar exudate production, which can result in more similar bacterial communities. Indeed, phylosymbiosis does not necessarily imply coevolution (Moran & Sloan 2015). The findings of the later chapters supported this hypothesis. *C. closterium* (Chapter III- V), *N. phyllepta* (Chapter V) and *S. robusta* (Chapter IV-V) could all be 'cured' of bacteria and remained healthy (i.e. actively growing) for at least a few weeks. Other studies have reported similar observations for other diatoms (Amin et al. 2015; Scholz 2014; Windler et al. 2014), although these studies also reported marked changes in morphology and a reduction of growth rates in the longer term (months). As axenic strains were not maintained for extended periods of time, we do not know if this would have been the case for the diatoms used in our studies. However, since diatoms can, at least briefly, remain healthy without bacteria, the diatom-bacteria associations could be ephemeral or sufficiently loose for host and bacteria to be interchangeable, reducing the opportunity for coevolution. Observations of single

diatom cells without any attached bacteria support this statement (Baker et al. 2016; Kaczmarska et al. 2005). Since coevolved bacteria are presumed to be linked to their host's fitness (O' Brien et al. 2019), we tested the effects of own and foreign bacteria in chapter III. Although foreign bacteria tended to be more growth-inhibiting, the failure to match the phylogenetic position of several *Cylindrotheca* strains with their growth response towards bacteria (Chapter III) again suggested that the underlying mechanisms were different from coevolution.

In the absence of marked coevolution, diatom-associated bacterial community composition and the specificity of the interactions, observed in this thesis, makes most sense in the light of ecological sorting (i.e. selection): a process during which the environment (in our case the host and the culture conditions) filters out the bacteria that does not possess the traits required for survival in a specific (host and/or environmental) habitat and competitive interactions between organisms can modify community composition (Weiher & Keddy 1995). Indeed the general enrichment of certain bacterial taxa fits well within the idea of an environmental filter shaped by the hosts and the culture conditions. Ecological sorting does not require diatoms and associated bacteria to have consistently co-occurred together. In fact, it is possible that bacteria establish themselves in the microbiome of a diatom they have never occurred with before, a process which is referred to as ecological fitting (Agosta & Klemens 2008). Newly associated bacteria might have the required traits from an interaction evolved in response to a different host. The fitting of the plant soil bacterial community to the diatom cultures in chapter IV may be an example of such ecological fitting, in which bacterial adaptations to interact with plants (or terrestrial diatoms and other microalgae) may be useful to interact with the marine benthic diatoms.

Even if coevolution between diatoms and bacteria is absent or weak, evolution is still likely to be acting on diatoms and bacteria, and might therefore important role in the assembly process. Bacterial traits can be under selection if they enhance the establishment success or the overall fitness of bacteria in microbiomes (Ackerly 2003). In turn, the continuous colonization of the algal host by bacteria likely adds to the selective pressure on host traits (O' Brien et al. 2019). The presence of such adapted traits in both partners, i.e. coadaptions, would help to explain some observations made in chapters III and IV. In both chapters, foreign bacteria tended to be more growth-inhibiting to a host than the host's own bacteria, at least if those bacteria naturally co-

occurred with their host (chapter IV). These observations suggest that adaptations are present in naturally co-occurring partners, resulting in reduced competition with the host, whereas such adaptations were probably absent in none naturally co-occur organisms. As we did not measure the fitness of the bacteria, we do not know if these adaptations also resulted in additional benefits for the bacteria when associated with specific hosts. The feedback between the establishment success of the organisms and the adaptations involved, illustrate well the complex eco-evolutionary dynamics in which interacting organisms are often involved (De Meester et al. 2019).

As we did not perform (meta)genomic, (meta)transcriptomic or metabolomic analyses, we can only speculate about bacterial and/or diatom host adaptations relevant to the assembly process. Recognition mechanisms to detect (suitable) partners may be complex, probably involving at least several signalling pathways in the diatom host (Duram et al. 2017). Amin et al. (2012) hypothesized that quorum sensing related mechanisms might be involved due to their prevalence in bacteria groups that are known to associate with diatoms and their relevance in other inter-kingdom interactions (e.g. with macroalgae, Trait et al. 2009). While its role as a signalling or recognition mechanism in diatom-bacteria interactions is not yet resolved, recent work has shown that quorum sensing can be highly relevant for the interactions between benthic diatoms and bacteria in other ways (e.g. quorum quenching by a benthic diatom (Syrpass et al. 2014); algicidal effects of a quorum sensing derivative (Stock et al. 2019)). Other potential molecular adaptations may be related to the release of amino acids (Paul et al. 2013), interference with the pheromones produced by the diatoms (Amin et al. 2012; Cirri et al. 2018) as well as to the production of other molecules such as dimethylsulfoniopropionate (DMSP) and indole-3-acetic acid (IAA) released by planktonic diatoms and bacteria respectively (Cooper & Smith 2015). As DMSP and IAA production have also been reported in coastal marine sediments (Crump et al. 2018; van Bergeijk et al. 2002), mechanisms involving these compounds could also be relevant in a benthic context.

Differential responses of benthic marine diatoms to bacteria

Most of the studies in this thesis focussed on the bacteria associated with the diatom strains belonging to the *C. closterium* and *S. robusta* species complexes (Appendix II; De Decker et al. 2018). These diatoms are intensively used as model organisms for a.o. ecophysiology (e.g. Moreno-Garrido et al. 2003) and life cycle regulation in

diatoms (e.g. Moeys et al. 2016) respectively, and have also previously been used to study interactions between diatoms and bacteria. Growth of *Cylindrotheca* species (*C. fusiformis* and *C. closterium*) was affected by the presence of different bacteria, both positively and negatively (Doiron et al. 2012; Grossart 1999; Han et al. 2016), and this depended on the environmental conditions (Grossart 1999). N-acyl homoserine lactones have been shown to induce biofilm formation in *Cylindrotheca* (Yang et al. 2016), whilst Doiron et al. (2012) showed a strong adhesion of bacteria to *C. closterium*. In response to the presence of bacteria, *C. fusiformis* increased its polysaccharide production (Guerrini et al. 1998). Several bacteria were shown to alter the growth of *S. robusta* and reduce its sexual reproduction rate (Cirri et al. 2018). These examples illustrate both the plethora of interactions in which these diatoms and associated bacteria can be involved, but more importantly, also confirm the potential for both these organisms as models to study benthic diatom-bacteria interactions.

We observed important differences between *C. closterium* and *S. robusta* in their response to bacteria (Chapter IV & V). Most notably, the interactions between *S. robusta* and bacteria were generally less negative (and more often even positive) for the growth of the host than those between *C. closterium* and bacteria. Although both diatoms can occur sympatrically, differences in their habitat preferences suggest that they differ in their ecophysiology. *S. robusta* is commonly found in less dynamic subtidal marine biofilms (De Decker et al. 2018), which will be relatively static and perhaps nutrient-poor. *C. closterium* on the other hand is found in diverse habitats, sometimes dominating older biofilms in tidal mudflats, but also occurring on ship hulls (Doiron et al. 2012) and inside sea-ice (Von Quillfeldt et al. 2003), in pelagic aggregates (Najdek et al. 2005) and on invertebrates (Gárate-Lizárraga & Esqueda-Escárcega 2016; Magesky et al. 2017). This might indicate a more flexible or perhaps even opportunistic nature of *C. closterium* as compared to *S. robusta*. In chapter V, we argued that, as was observed for plants (Lemmermeyer et al. 2015), a more opportunistic, fast-growing life strategy might be associated with more negative bacterial interactions. In addition, *C. closterium* has repeatedly been reported to be mixotrophic (Darley et al. 1981; Mensens et al 2017; Vanelslander et al. 2009). The generally more negative interactions between bacteria and *C. closterium* could therefore also result from competition for organic carbon, which might not be occurring with *S. robusta*. Other differences, such as in differences in exudate composition

between diatom species, may also lie at the basis of the observed differences (Dadglio et al. 2018; Mühlenbruch et al. 2018; Wear et al. 2015). As exudates were not characterised or quantified in this thesis, we cannot make any statements on this.

Ecological relevance

With this work, we have provided novel insights in the interactions between marine benthic diatoms and heterotrophic bacteria. These interactions are occurring in many marine phototrophic biofilms, the extensive biofilms on the surface of intertidal mudflats being the most notable ones (Stal & Brouwer 2003). Intertidal mudflats are extremely valuable ecosystems (Costanza et al. 2014), which is to some extent due to these, generally diatoms and bacteria dominated (Underwood & Kromkamp 1999) biofilms which enhance nutrient fluxes (Cook et al., 2009) and provide autochthonously fixed carbon to the successive trophic levels within the mudflat (Moerdijk-Poortvliet et al., 2018). Our findings directly contribute to better understanding of the biological processes that the structure these biofilms and can potentially even help to predict the effects of global change or the introduction of invasive microorganisms (Taylor & Bothwell 2014) on the functioning of the biofilm community in intertidal mudflats.

More broadly, this fundamental research was aimed at understanding the underlying processes that determine the structure and composition of a host associated microbiome. Although bacterial habitats such as our skin or the rhizosphere of our crops vary substantially from a diatom culture, the fundamental ecological processes (dispersal, local diversification, environmental selection, and ecological drift) that structure microbiomes (Costello et al. 2012; Vellend 2010) are the same across different habitats. By identifying these processes in our (easy to manipulate) study system, our findings can help to understand the similarities and differences across bacterial communities associated with different hosts. Furthermore, a better insight in these processes can help us to better control host associated microbiomes, resulting in improved host benefits, such as disease resistance or improved yields (Panke-Buisse et al. 2015; Weese, 2013).

In our studies, we worked in a very standardized and controlled experimental setting. One of the major challenges of most lab-based studies is to link the experimental observations made in the lab to *in situ* patterns and processes. Field observations have repeatedly shown correlations between the structure and abundance of the

microphytobenthos and heterotrophic bacteria in tidal flats (Bolhuis et al. 2013; Decleyre et al. 2015; Lavergne et al. 2017), but these approaches do not allow untangling the underlying interaction mechanisms. As argued above, the multiwell environment could have had a non-negligible effect on our artificial consortia (Chapter IV; Crenn et al. 2018). The interactions we observed could be significantly different in a more complex and dynamic abiotic (sediment substrate, microscale biogeochemical gradients) and biotic (grazers, parasites, dispersal, etc.) tidal flat environment. Nonetheless, by increasing both diatom and bacterial diversity (Chapters III-V) instead of the one-on-one interactions, used in many diatom-bacteria studies (e.g. Brucker et al. 2011), and by considering an ecologically relevant context where possible (e.g. own versus foreign bacteria in Chapter III or naturally co-occurring versus not naturally co-occurring in Chapter IV) we attempted to provide a framework that would allow us to project our results to the *in situ* situation.

Most of the observed interactions between bacteria and diatoms in this thesis resulted in neutral or (slightly) negative effects on the diatoms, particularly with *C. closterium* (see above). This was in marked contrast with what was reported for the diatoms *Haslea ostrearia* (Lépinay et al. 2018), where diatom growth rates were on average 58% higher with bacteria than without, and *Pseudo-nitzschia multiseries*, where over half of the bacterial isolates tested promoted diatom growth (Amin et al. 2015). The differences between our findings and these studies may relate to host identity and/or culture conditions, but may also relate to the specific habitat we studied (marine inter- and subtidal biofilms). We argue that especially in tidal flats the dependency of bacteria on diatoms and vice versa might be less pronounced, resulting in less positive interactions. Pelagic systems tend to more nutrient deplete, resulting in tighter and more obligatory interactions between microalgae and bacteria (Buchan et al. 2014; Geng & Belas 2010), in which the algae depend on nutrient remineralization by heterotrophic bacteria and bacteria depend on the organic carbon produced by the phytoplankton (Sarmento & Gasol 2012). In tidal flats, nutrients are generally more abundant (Reckhardt et al. 2015) and a significant portion of the organic carbon might be allochthonous, derived from terrestrial or pelagic sources (Cook et al. 2004).

Due to the high bacterial and algal diversity in tidal flats (Forster et al. 2006; Kim et al. 2004; Chapters II, IV&V), studies about one-on-one interactions may be less ecologically relevant. In chapter III, where we mostly worked with bacterial isolates to

study one-on-one interactions with diatoms, we showed that a natural bacterial community can rapidly outcompete a single species. Furthermore the structure of the *C. closterium* microbiomes (Chapter II) suggested important interactions between the bacteria themselves (cf. also Dittmann et al. (2018) for *Emiliania huxleyi* microbiota), which can further modulate host-bacteria interactions. In chapter V, we tested the effect of increasing diatom diversity (albeit still at very low levels). While the bacterial communities in mixed diatom cultures could be predicted from their composition in the diatom monocultures, the differential effect of the bacteria on diatom growth resulted in substantial shifts in diatom community composition. Our results thus confirm that the effects of diatom-bacteria associations should ideally be considered in a more diverse context. If not, the effects of diversity on these associations or *vice versa* could be overlooked.

Limitations and future perspectives

We recognize that we could not consider several other relevant aspects of benthic diatom-bacteria interactions, due to practical, methodological or time constraints. Here we note a few of the most important limitations, and more importantly, provide suggestions for future studies to overcome these shortcomings.

Imaging-PAM fluorometry (Chapters III-IV) and 16S rDNA HTP sequencing (Chapters II, IV & V) were both extensively used in this thesis. The reliability of Imaging-PAM fluorometry was assessed in Appendix I. This method indeed proved to be a suitable method to assess diatom growth. However due to fluorescence differences between exponentially and stationary diatom cultures and the potential underestimation of stationary cultures due to the clustering of dense cultures, we could not reliably compare algal densities of the stationary cultures. This does imply that we were mostly limited to detecting differences between treatment when nutrients were relatively abundant. We are aware that there might have been relevant interactions in the stationary growth phase of the diatoms (e.g. Bruckner et al. 2008; Grossart 1999) and that effects, such as bacterial remineralisation or increased resource competition between bacteria and their host, may thus be unnoticed. In future studies, diatom growth (during the exponential phase) by means of Imaging-PAM fluorometry could be complemented with cell counts of the stationary cultures.

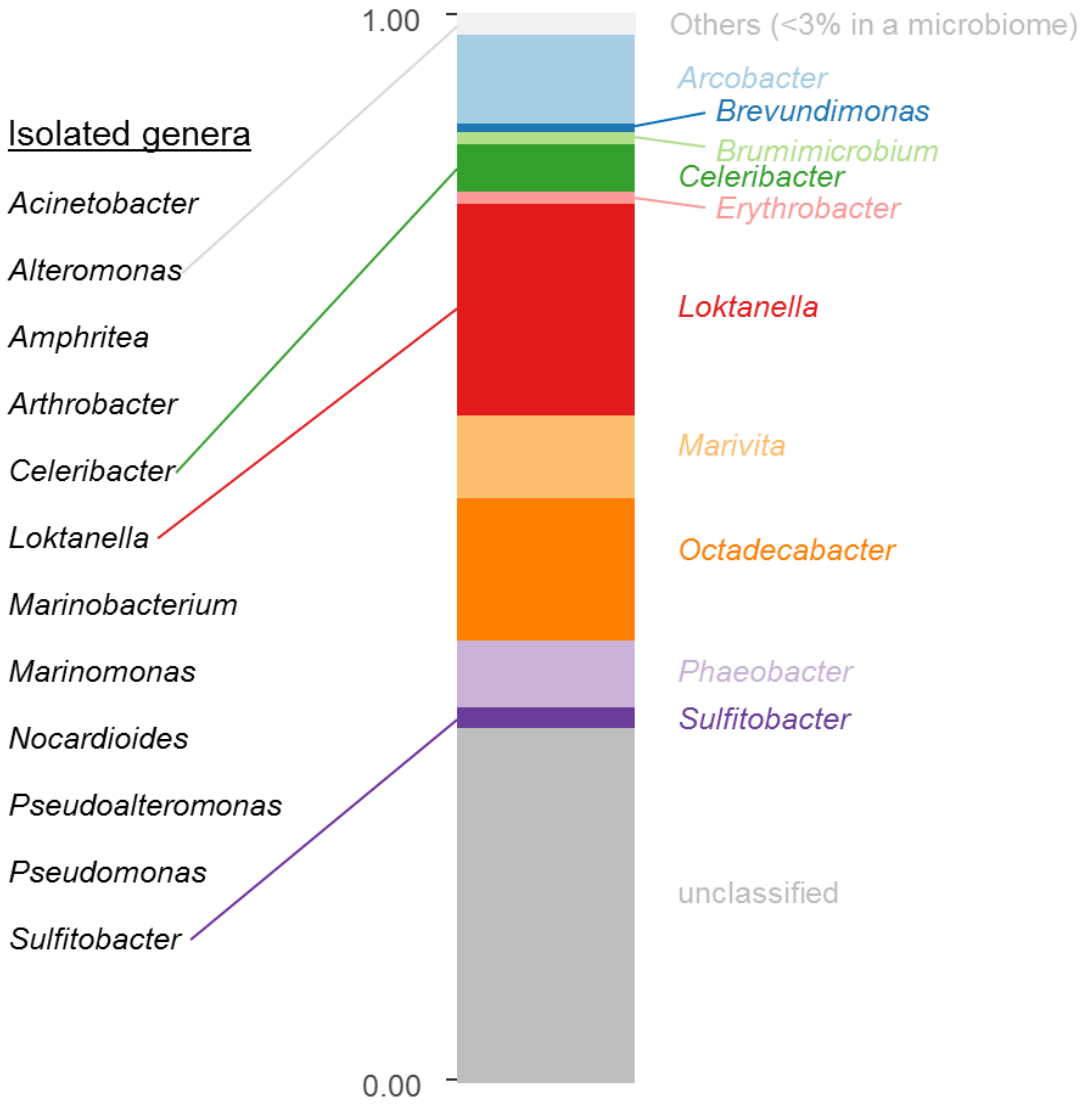


Figure 3: Differences between bacteria identified by 16S rDNA HTP sequencing and by isolation. The bacteria genera isolated from six *C. closterium* strains in chapter III are listed on the left, the relative abundances of bacterial genera identified by 16S rDNA HTP sequencing on those same six *C. closterium* strains is given on the right. Bacterial genera which have been identified in the same *C. closterium* strain by both methods are connected.

16S rDNA HTP sequencing has its own limitations, such as PCR biases and sequencing errors (Schirmer et al. 2015; Wen et al. 2017). The comparison between replicated samples showed that the sequencing was reasonably consistent in a sample and the inclusion of mock communities suggested that most organisms present in a sample could readily be detected. The comparison with the isolates, obtained from six *C. closterium* strains in chapter III, with the sequencing of those same diatom strains (Chapter II) did however reveal marked differences between both methods (Figure 3). As the samples for sequencing were taken only a few weeks before the cultures were

used for bacterial isolation, strong temporal shifts or bacterial contaminations were unlikely. Whilst these pronounced differences between HTP sequencing and isolation are not new (Tytgat et al. 2014), this might have implications on the relevance of our findings. Most importantly, relevant bacterial groups might still have been missed by the sequencing (e.g. *Marinobacter*) and, as a result species- or strain specific bacteria might have been missed. This problem could be resolved in future studies using additional primers, as for instance in Dziallas & Grossart (2011). In order to obtain more bacterial isolates, representative for the diatom-associated bacterial communities, novel cultivation approaches could be applied including microfluidic systems (e.g., Pan et al. 2011) and cultivation chips (Nichols et al. 2010).

To characterise the bacterial communities, we consistently focussed on total bacterial communities associated with late-exponential to stationary cultures. Whilst we are aware that the algal growth phase has important effects on the diatom-associated bacterial community (Baker et al. 2016) and that there might be strong differences between free-living and attached bacterial communities (Grossart et al. 2005), analysing these factors, in addition to all other factors of interest, was beyond the scope of this work. By also focussing on growth phase or the different fractions of the bacterial community, it might be possible to verify when and where species- or strain-specific differences are most pronounced. This might therefore be a useful approach for a dedicated study in the near future. Other potentially relevant factors, such as temperature (Dziallas & Grossart 2012) also remain interesting to include in future studies.

The study of long-term dynamics between diatoms and their associated bacteria is interesting to infer stability or species-turnover in these communities. In chapters III & V, the experimental duration was limited to one week (one growth cycle) and in chapter IV to eight growth cycles in total. Based on the findings in chapter IV, one week was relatively short and probably too short for interactions to become stable, perhaps even with the bacterial isolates (Chapter II). Many one-on-one diatom-bacteria interaction studies have a limited experimental duration, often one growth cycle (e.g. Bruckner et al. 2008; Sison-Mangus et al. 2014). In studies with complete bacterial communities (e.g. Ajani et al. 2018; Eigemann et al. 2013), community dynamics over growth cycles have also largely been ignored. The studies that did focus on these dynamics have reported contrasting findings: Sapp et al. (2007a) reported marked shifts in the

bacterial community whilst Behringer et al. (2018) and Schäfer et al. (2002) showed these communities to be relatively stable. Whilst there are marked differences between these experiments, most notably the time windows considered, our results (chapter IV) are in agreement with Sapp et al. (2007a). Future studies might therefore benefit from including additional growth cycles in order to gain more insight in the long-term effects of bacteria-diatom associations.

We have worked with different bacterial inocula throughout this thesis (Chapter III-V). The initial diatom growth response differed from neutral (chapter III) to negative (IV-V) in response to these inocula. The bacterial communities differed between studies in terms of how they were extracted from the natural samples (neutral red treatment in Chapter IV; additional filter step in Chapter V as compared to Chapter III), but also due to differences between the natural samples, hence hampering comparisons between studies. Due to the spatio-temporal and natural variation in *the in situ* bacterial communities, experiments relying on these communities are never completely reproducible. Furthermore it is difficult to fully characterize a natural inoculum. For instance, we did not identify the viruses and archaea in these inocula, whilst we could expect, based on *in situ* observations, these groups to impact the diatoms - bacteria dynamics (Montanié et al. 2015; Needham & Fuhrman 2016; Steele et al. 2015). Future studies that rely on natural inocula could benefit from characterising the virome and archaeal community. On the other hand, evidence for the presence of these groups in cultures is largely lacking which suggests that viruses and archeae might not be able to persist under culture conditions (Dziallas & Grossart 2011). Nonetheless, we suggest that future studies could use a synthetic bacterial inoculum to circumvent these problems. The inoculum could be composed of isolated bacteria that can be selected based on the observations from previous studies, including this thesis.

For future studies, focussing on the identification of (co)adaptions relevant in the assembly process of a benthic diatom-associated bacterial community, we suggest considering a metatranscriptomic/metabolomics approach. These ‘omics’ techniques are becoming increasingly accessible and could fill important gaps in our understanding of diatom-bacteria associations. For instance, to confirm if selective bacterial enrichment during the assembly is accompanied by a functional enrichment or to identify which adaptations in both diatoms and bacteria were at the basis of the observed specificity. The ongoing effort to sequence genomes of diatoms (e.g. Traller

et al. 2016) and diatom associated bacteria (e.g. Töpel et al. 2018 & 2019) is likely to be of great value for the assembly and annotation process of such metatranscriptomic/metabolomics approaches. Also the use of metagenomics on diatoms cultures with their associated bacteria can help identify relevant adaptations by reconstructing metabolic pathways between the organisms (i.e. interactomes; Garcia et al. 2015). The use of 'omics' techniques also opens up the avenue to study the interactions between the bacteria themselves. In this thesis, similar to most studies so far, the emphasis has been on diatom-bacteria interactions, but as evidenced from our results (potentially positive interactions between bacteria in Chapter II, effect of natural inoculum on *Marinobacter* in Chapter III) and previous studies (e.g. Majzoub et al. 2019), the effects between bacteria themselves should also be considered.

Finally, we attempted to make the experimental setup more ecologically relevant by increasing the number of algal species. Since we showed that the differential responses to bacteria between diatom species can have important ecological implications (Chapter V), it would be interesting to include even more marine benthic diatoms in future experiments, preferably with differ growth rates and ecology to test the relevance of such differences. It would definitely be interesting to also include diatoms that are known to produce biocides such as *Nitzschia cf pellucida* (Vanelslander et al. 2012).

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Summary

samenvatting

Summary

Diatoms are one of the most successful and species-rich groups of eukaryotic microalgae in today's oceans. Like many organisms, they live in close association with bacteria. While such diatom-bacteria consortia have been regularly studied in marine planktonic diatoms, little is as yet known about such associations and their interactions in marine benthic habitats. In marine tidal sediments, benthic diatoms and bacteria live in close proximity in densely packed biofilms. These consortia play a crucial role in the functioning of tidal flats by regulating nutrient fluxes across the sediment-water interface, fuelling coastal food webs and stabilizing the sediment. Despite their importance, the processes that drive community assembly and shape the interactions between the bacteria and diatoms in these biofilms are not well understood. In this thesis, we aimed to get a better understanding of the interactions driving bacterial community assembly when associated with two marine benthic diatoms, *Cylindrotheca closterium* and *Seminavis robusta*, and the effect that bacteria have on the fitness and functioning of their diatom host.

In a first study we examined the structure and composition of the bacterial community in recently associated *C. closterium* strains. The effect of several of these bacteria on their host was assessed by means of one-on-one cocultures in a second study. The assembly process of the bacterial communities associated with C. *closterium* and *S. robusta* was monitored in a third study, where we also evaluated if the assembled communities would specifically benefit their host by transplanting the assembled bacterial communities between diatoms. In a forth, and final experiment, we evaluated the impact of the interactions between bacteria and benthic diatoms on the structure and the functioning of a benthic microbial community.

The bacterial communities, associated with the recently isolated *C. closterium* strains, were dominated by Alphaproteobacteria, Gammaproteobacteria, Epsilonproteobacteria and Flavobacteriia. Bacteria from the *Roseobacter* group were particularly prevalent and therefore likely to be well adapted to associate with *C. closterium*. As a result of the differential selection between the diatoms, the structure of the bacterial communities correlated to the phylogenetic relationship of the hosts. In addition, neutral processes resulted in a lasting imprint of the environment from where the diatoms were isolated.

The assembly of a bacterial community around a benthic diatoms was accompanied by a selective enrichment of the same bacterial classes observed in the first study. In the initial stages of the assembly process, the growth of the diatoms was suppressed, most probably as a result of competition between the bacteria and diatoms. Diatom growth gradually recovered, which coincided with the development of diatom species-specific bacterial communities. By transplanting bacterial communities between diatom hosts, we showed that a bacterial community could have a less negative effect on the growth of its host if it was phylogenetically more similar to the previous host of that community. A similar observation was made in the one-on-one cocultres whereby own bacteria were less growth-inhibiting than foreign bacteria. These results showed, that under the right circumstances, host-specificity can result in a reduced competition with its host.

Lastly, to evaluate the effect of the associations between bacteria and benthic diatoms in a more complex community, we grew three different diatom species, as monoculture and cocultures, with and without bacteria. The effect of bacteria on diatom growth was again species-specific, resulting in compositional shifts when different diatom species were grown together, which suggests that interactions between bacteria and diatoms residing in mudflats are an important factor in the structuring of the benthic microbial community composition.

Together, our results contribute to a better understanding of the interactions between bacteria and diatoms in tidal flats and the ecological relevance thereof. Our findings suggest that bacteria and diatoms have adapted to associate with specific partners, resulting in the high levels of specificity repeatedly observed in this thesis. By identifying these underlying adaptations, we could gain a better insight into the evolutionary dynamics between benthic diatoms and their associated bacteria. With the genomes of *C. closterium* and *S. robusta* soon available, these benthic diatoms could serve well in a transcriptomie-based approach to detect such adaptations.

Samenvatting

Diatomeeën, ook wel kiezelwieren genoemd, zijn één van de meest succesvolle en soortenrijke eukaryote microalgen in de oceaan. Zoals vele organismen leven ze in nauwe associatie met bacteriën. Hoewel dergelijke diatomeeën-bacterieconsortia al vaker zijn bestudeerd in de open oceanen, is er nog weinig geweten over dergelijke associaties in mariene bentische habitats. In de sedimenten van marine intergetijdengebieden leven benthische diatomeeën en bacteriën dicht bij elkaar in dense biofilms. Deze consortia spelen een cruciale rol bij het functioneren van de intergetijdengebieden door de nutriëntenfluxen tussen sediment en water te reguleren, als voedselbron voor de hogere trofische niveaus te fungeren en door het sediment te stabiliseren. Desondanks hun economische en ecologische relevantie, zijn de onderliggende processen die de benthische diatomee-bacterieconsortia vormgeven nog niet goed gekend. In dit proefschrift hebben we gepoogd om meer inzicht te krijgen in de interacties tussen twee mariene benthische diatomeeën, *Cylindrotheca closterium* en *Seminavis robusta*, en hun bacteriën.

In een eerste studie onderzochten we de structuur en samenstelling van de bacteriële gemeenschap in recent geïsoleerde *C. closterium* stammen. Het effect van deze bacteriën op hun gastheer werd in een tweede studie getest door middel van één-op-één co-culturen. Het ontwikkelen van de bacteriegemeenschappen rond *C. closterium* en *S. robusta* werd gevolgd in een derde studie, waarbij we eveneens onderzochten of de nieuw samengestelde gemeenschappen hun gastheer zouden bevoordelen door de gemeenschappen tussen diatomeeën uit te wisselen. In een vierde en laatste experiment evalueerden het belang van de interacties tussen bacteriën en benthische diatomeeën voor het structureren van de microbiële gemeenschappen *in situ*.

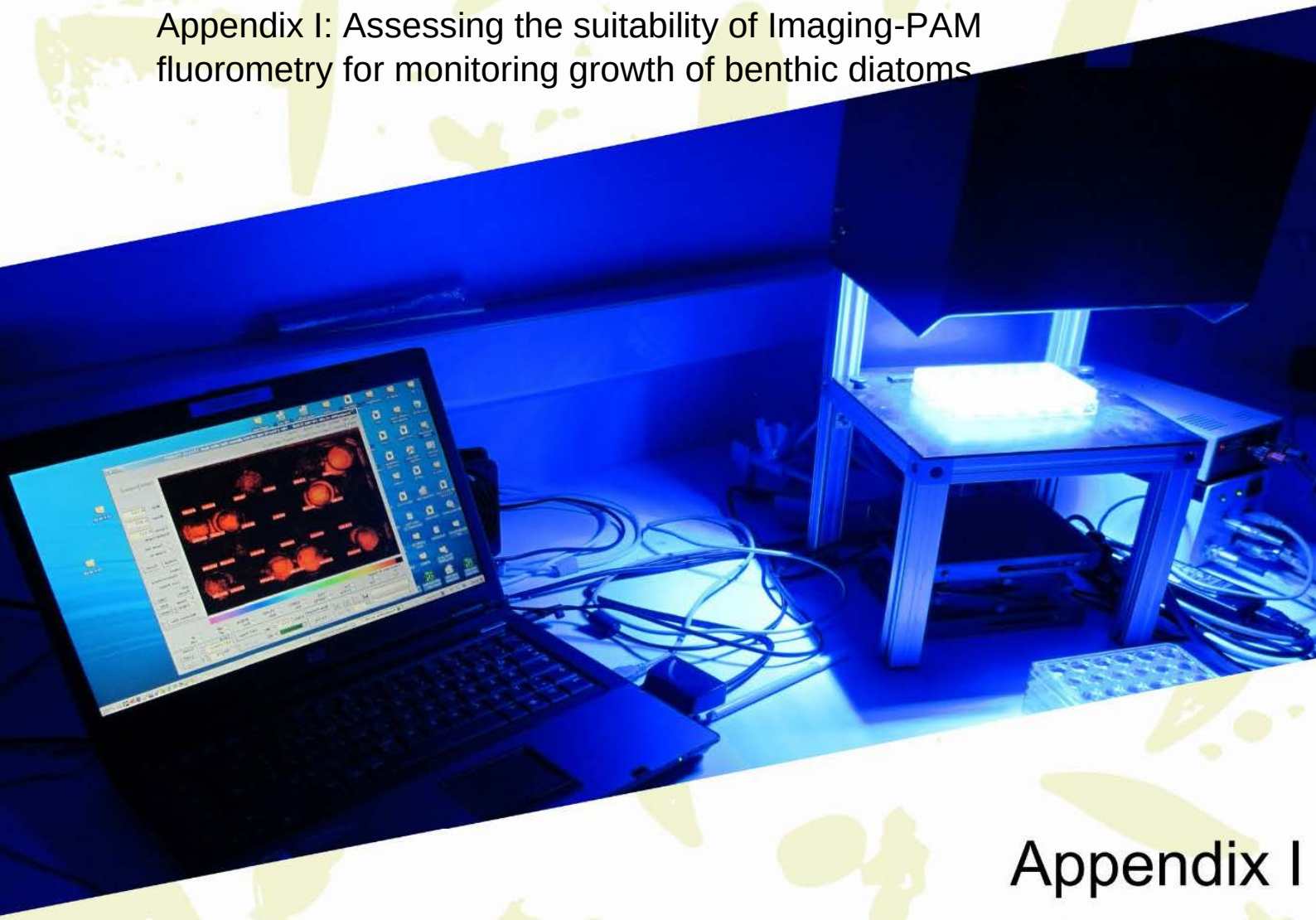
De bacteriële gemeenschappen, geassocieerd met de recentelijk geïsoleerde *C. closterium* stammen, werden gedomineerd door Alphaproteobacteria, Gammaproteobacteria, Epsilonproteobacteria en Flavobacteriia. Bacteriën van de *Roseobacter*-groep waren heel aanwezig en zijn waarschijnlijk dus goed aangepast aan *C. closterium*. Vanwege de differentiële selectie door de diatomeeën, correleerde de structuur van de bacteriële gemeenschappen met de fylogenetische relatie tussen de gastheren. Het effect van de omgeving van waaruit de diatomeeën werden geïsoleerd bleef merkbaar door de invloed van neutraal ecologische processen.

Het ontwikkelen van de bacteriële gemeenschap rond een benthische diatomee ging gepaard met een selectieve aanrijking van dezelfde bacteriële groepen die in de eerste studie werden waargenomen. Initieel werd de groei van de diatomeeën onderdrukt, hoogstwaarschijnlijk als gevolg van competitie tussen de bacteriën en diatomeeën. Later herstelde de groei van de gastheer zich, wat gepaard ging met het ontwikkelen van een gastheer-specifieke bacteriële gemeenschap. Door gemeenschappen uit te wisselen tussen de verschillende diatomeeën, toonden we aan dat de bacteriële gemeenschap vaak minder groei-inhiberend was als de gastheer nauwever verwant was aan de vorige gastheer. Op basis van de in de één-op-één co-culturen vonden we iets gelijkaardigs, aangezien de eigen bacteriën minder groei-inhiberend waren dan bacteriën van een andere gastheer. Deze resultaten suggereren dat, onder de juiste omstandigheden, gastheer-specifieke bacteriën minder zullen concurreren met de gastheer.

Ten slotte hebben we het effect van de interacties tussen bacteriën en benthische diatomeeën in een meer diverse gemeenschap getest. Hiervoor hebben we drie verschillende soorten diatomeeën gekweekt, als monocultuur en co-culturen, met en zonder bacteriën. Het effect van bacteriën op de groei van diatomeeën was opnieuw soortsspecifiek en resulteerde daardoor in een verandering van de soortssamenstelling wanneer verschillende diatomeeënsoorten samen werden gekweekt, wat suggereert dat de interacties tussen bacteriën en diatomeeën belangrijk zijn voor het structureren van de microbiële gemeenschap in intertidale sedimenten.

Samen dragen onze resultaten bij tot een beter begrip van de interacties tussen bacteriën en diatomeeën in sedimenten van marine intergetijdengebieden. Onze bevindingen suggereren dat bacteriën en diatomeeën zich hebben aangepast om te associëren met specifieke partners, resulterend in de hoge niveaus van specificiteit die herhaaldelijk in dit proefschrift worden waargenomen. Door deze onderliggende adaptaties te identificeren, kunnen we een beter inzicht krijgen in de evolutionaire dynamiek tussen benthische diatomeeën en hun geassocieerde bacteriën. Met de genomen van *C. closterium* en *S. robusta* binnenkort beschikbaar, zouden deze soorten uiterst geschikt zijn om, aan de hand van transcriptoom studies, deze adaptaties te detecteren.

Appendix I: Assessing the suitability of Imaging-PAM fluorometry for monitoring growth of benthic diatoms



Appendix I

Assessing the suitability of Imaging-PAM fluorometry for monitoring growth of benthic diatoms

Published as: Stock, W., Blommaert, L., Daveloose, I., Vyverman, W., & Sabbe, K. (2019). Assessing the suitability of Imaging-PAM fluorometry for monitoring growth of benthic diatoms. *Journal of Experimental Marine Biology and Ecology,* 513, 35-41.

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Abstract

High-throughput monitoring of growth using Pulse Amplitude Modulated (PAM) chlorophyll fluorescence imaging (Imaging-PAM) is an important tool for experimental ecological and physiological research on benthic diatom cultures, as due to substrate adhesion non-destructive subsampling for biomass determination is difficult and/or not representative. We evaluated the reliability of Imaging-PAM for quantifying benthic diatom biomass by comparing biomass determinations based on PAM fluorescence parameters to those based on cells counts and chlorophyll *a* measurements for cultures of the diatoms *Cylindrotheca closterium* and *Seminavis robusta* in different growth phases (exponential vs stationary) and cell size ranges. We found that PAM fluorescence imaging is particularly suited to monitor growth during the exponential phase. The dark adapted minimal fluorescence (F_0) parameter proved to be the most reliable fluorescence-based biomass proxy, especially for establishing complete growth curves, as it was the most robust parameter to growth phase changes. A dark adaptation period prior to the PAM measurements improved the quantification of the stationary but not of the exponential cultures. Under the right conditions, dark adaptation can thus be omitted for exponentially growing cultures to enhance throughput. The use of multiwell plates for growth monitoring with the Imaging-PAM is feasible even though there can be a slight biomass underestimation near of the edges of the plate. Based on these findings, we formulate specific guidelines for using Imaging-PAM fluorometry in experiments with benthic diatom cultures. We especially recommend taking caution when working under conditions which potentially influence the condition of the photosynthetic apparatus of the algae

Introduction

Monitoring growth is an essential component of most experimental ecological and physiological research on microalgae (Litchman and Klausmeier 2008, Kassim & Meng. 2017, Tamburic et al. 2014). While planktonic species can easily and non-destructively be subsampled and quantified using various tools, ranging from microscopic counting chambers to flow cytometry (Behrenfeld et al. 2006), non-intrusive subsampling is much harder for benthic species which adhere to substrates and/or form biofilms. In addition, growth monitoring of benthic species in a high number of experimental treatments requires a reliable high-throughput method.

The use of in vivo chlorophyll *a* fluorescence measurements with Pulse Amplitude Modulated (PAM) fluorometry has been proposed as a method to quantify biomass in phototrophic microorganisms (Karsten et al. 1996), including benthic diatoms (Serôdio et al. 1997; Honeywill et al. 2002). This technique relies on the emittance of red to far-red light mainly by photosystem (PS) II (Krause and Weis 1991) upon excitation by light with a shorter wavelength.

Two main fluorescence parameters are commonly measured; these differ in the oxidation state of the primary electron acceptor in PSII, quinone acceptor Q_A (Baker 2008). After adapting a sample to darkness, Q_A becomes maximally oxidized. The PSII reaction centers can then use the captured light energy to reduce Q_A and are referred to as being 'open'. When weak measuring light pulses are now applied (in order to keep the Q_A pool maximally oxidized), the fluorescence yield of these pulses will be minimal (F_0). However, when the sample is exposed to a very strong light pulse (called a saturating pulse), Q_A will become fully reduced and PSII reaction centers will not be able to perform additional charge separations (i.e. they are 'closed'). As a result, the fluorescence yield will now reach a maximal level, F_m (Consalvey et al. 2005).

F_0 is more commonly used than F_m as a biomass estimator as it appears to correlate better with Chl *a* content (Serôdio et al. 2001; Honeywill et al. 2002).The difference between minimal and maximal fluorescence (F_m-F_0) is the variable fluorescence, F_v. When F_v is divided by F_m, we obtain the maximal quantum yield of PSII (Baker 2008), as it reflects the relative amount of fluorescence that is quenched by the PSII reaction centers. A decrease of the F_v/F_m may signal an inhibition of photosystem II quantum yield and is for example used to identify phototoxic compounds (Schreiber et al. 2007).

Exposure of algal cells to high light alters the oxidation state of Q_A. In addition, high light exposure can induce non-photochemical quenching of chlorophyll fluorescence (NPQ, Jesus et al. 2006). To prevent this, a dark or low light adaptation period prior to the PAM measurements has been suggested (Honeywill et al. 2002, Consalvey et al. 2004, Jesus et al. 2006). A dark adaptation period of 15 minutes was suggested based on in-situ experiments (Serôdio et al. 1997; Honeywill et al. 2002). It is currently unknown if such a dark adaptation period is useful for cultures under laboratory conditions.

Different PAM fluorometers exist. The Imaging-PAM fluorometer is equipped with a CCD camera which allows recording a reasonably large area (up to 10 × 13 cm in the case of the Walz Imaging system used in this study). It is particularly useful for imaging multiwells or cell culture flasks (Schreiber 2004), and hence monitoring the growth of benthic microalgae. The spatially explicit recording of the fluorescence signal makes it possible to distinguish the fluorescence of different wells of a single plate (Fig. 1). At the same time, the fluorescence assessment over the entire well area allows correction for potentially heterogeneous occurrence of algae within the well, typical for many benthic algae. On the downside, fluorescence cross-talk between neighbouring wells can influence the readings.

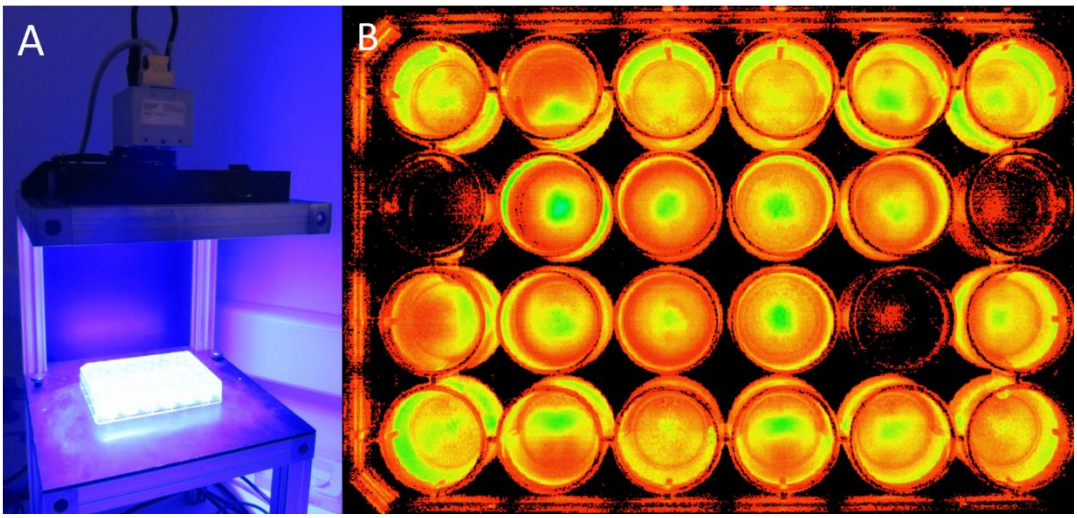

Figure 1: Imaging PAM fluorometry applied on a 24well plate. (A) shows an Imaging-PAM fluorometer (IMAG-MAXI; Walz, Germany) when applying a light pulse. The blue light is emitted by the leds in the black panel on top and the fluorescence is recorded by the camera mounted in the middle of the panel. The recording can be visualized as a false colour image (B) in which increasing fluorescence is shown as a colour shift from red to green over yellow. Notice that the microalgae in these wells are usually denser in the center and the fluorescence crosstalk from wells with high biomass in those with a low biomass. The false colour image was generated by ImagingWin v2.46i (Walz, Germany).

Imaging-PAM fluorometry has been used in a wide variety of applications, including quantification of algal biofilms on building materials (Eggert et al. 2006), photobiology of corals (Ralph et al. 2005), photoprotection of microalgae (Serôdio et al. 2012) and the evaluation of toxicity of specific compounds in algal cultures (Magnusson et al. 2010). Although Imaging-PAM fluorometry has also repeatedly been used to compare growth of algal cultures in the lab (e.g. Stock et al. 2018), its suitability for this application has not yet been rigorously evaluated. Under such conditions, i.e. unialgal cultures supplemented with a growth medium and maintained in moderate light (as is the case for most cultivation based microalgal studies), Imaging-PAM fluorometry is much more straightforward than in most in-situ situations, where it is convoluted by the taxonomic composition of the algal community (Vieira et al. 2013) as well as attenuation of the fluorescence signal by the matrix (usually sediment) (Morelle et al. 2018).

In order to use PAM fluorometry-based parameters as reliable growth estimators, changes in these parameters need to reflect proportional changes in biomass. It is therefore essential to know under which conditions this proxy correlates linearly with biomass and which factors influence the linearity of this relation. In addition to the experienced light climate, other factors may influence the physiological state of PSII and hence the reliability of F_0 or F_m as biomass proxies (Perkins et al., 2010; Consalvey et al. 2004; Jesus et al. 2006). Stationary cell cultures may have an altered PSII photophysiology (Forster and Martin-Jézéquel 2005; Bender et al. 2014) as nutrients essential to PSII, such as iron, become limited. In addition, the Chl *a* content per cell may shift during nutrient limitation, although this may be compensated for by an increased fluorescence per unit of chlorophyll (Kruskopf and Flynn 2006). In addition, extended lab cultivation might influence algal characteristics. This certainly is the case for diatoms, which display a cell size reduction cycle during vegetative growth, resulting in a gradual decrease of the mean population cell size (Round et al. 1990). Since smaller cells typically contain less Chl *a* (Maraóón et al. 2007), cell size reduction may impact the relationship between cell counts and fluorescence-based biomass proxies. Finally, the above effects may differ between species (Juneau and Harrison, 2005).

Our aim was to assess the reliability of Imaging-PAM fluorometry as a high-throughput method to compare biomass in experiments with unialgal benthic diatom cultures. This included identifying factors which may influence the fluorescence signal, such as growth phase and cell size, as well as assessing the suitability of multiwell plates to obtain trustworthy fluorescence readings. We compared Imaging-PAM fluorometry-based parameters with light microscopy counts and Chl *a* for two different benthic diatom species, viz. *Cylindrotheca closterium* (Ehrenberg) Reimann & Lewin and *Seminavis robusta* Danielidis & Mann. F_0 and F_m were measured for a range of different cell concentrations and compared with (automated) cell counts and Chl *a* concentrations. This was done for cultures before and after dark adaptation in order to assess the degree of linear relation between the different proxies. To identify the effects of cell size and growth phase on PAM fluorometry, stationary and exponential cultures as well as different cell sizes were compared for the two diatom species. Both growth phase and cell size were hypothesized to impact the amount of fluorescence per cell as we expected a decreasing chl *a* content per cell with decreasing cell size and likewise a decrease in fluorescence per cell as the algal cultures progress from exponential to stationary phase. We expected similar trends in both species. All experiments were carried out in 24-well plates to simultaneously evaluate the presence of potential artefacts (e.g. fluorescence cross-talk between wells) of measuring fluorescence in multiwell plates.

Methods

Culture conditions

Seminavis robusta (DCG 0105) and *Cylindrotheca closterium* (DCG 0623) cultures were obtained from the BCCM/DCG diatom culture collection (http://bccm.belspo.be/about-us/bccm-dcg). Cultures were maintained in artificial seawater (ASW; PRO-REEF Sea Salt, Tropic Marin, Dr. Beiner GmbH, Wartenberg,

Germany) supplemented with Guillard's (F/2) Marine Water Enrichment Solution (Sigma-Aldrich, Germany) at 18°C with 12:12 light/dark period (20-25 µmol photons $m^{-2}s^{-1}$ from cool-white fluorescent tubes). Smaller cells for each of the species were obtained by several rounds of re-inoculation, which lead to a gradual cell size reduction. Culture batches were grown in 75 cm² Cellstar cell culture flasks (Greiner Bio-One, Austria). Both species typically grew in a benthic growth mode on the bottom of the culture flasks. Growth was monitored using PAM fluorometry, using F_0 as a biomass proxy (see below).

Experimental setup

Cultures were harvested by scraping (cell spatula; TPP, Trasadingen, Switzerland) in early exponential (2-3 days after diluting the culture in F/2 medium) and stationary (7 days after diluting the culture in F/2 medium) growth phase, to compare the effect of growth phase on PAM-based biomass proxies. The effect of cell size was only tested for exponentially growing cultures. Two sizes for each species were used: large and small *S. robusta* (frustule length of 43.14 ± 0.63 µm vs 25.99 ± 0.54 µm) and large and small *C. closterium* (frustule length of 35.44 ± 0.78 µm vs 24.70 ± 1.09 µm).

After harvesting, the medium was partially (at least 70%) refreshed: ASW+F/2 was used for exponentially growing cultures and ASW without F/2 for stationary cultures. A dilution series was obtained from every culture by vortexing and transferring subsamples of the culture to a transparent 24-well plate (Greiner Bio-One, Austria). Between 0 and 1.5 ml of the culture was subsampled; up to 48 subsamples were taken from a single culture. These were randomly dispensed over the wells. The volume in every well was then amended with medium to a final volume of 1.5 ml for all cultures. The cells were allowed to settle overnight under the above-mentioned culture conditions. The plates were exposed to growth light conditions for more than three hours (20-25 µmol photons $m^{-2}s^{-1}$) before PAM fluorometry measurements (see below), after which they were stored in the dark for 15 min. After dark adaptation, the cultures were again measured using PAM fluorometry. Subsequently, they were photographed for automated cell counting and then harvested for Chl *a* determination (see below). A schematic representation of the experimental setup can be found in the supplementary material (Supp. Fig. 1)

PAM fluorometry

PAM fluorometry was conducted with a Maxi Imaging-PAM *M-series* (Heinz Walz GmbH, Effeltrich, Germany), equipped with an IMAG-K4 camera and mounted with an IMAG-MAX/F filter to remove a fraction of red light, emitted by the blue LEDs, which could be reflected on the water surface or the lid of the well-plate and be detected by the camera. Heterogeneity of measuring light intensity was corrected for by recording a correction file (imaging of a sheet of white paper) as recommended by the manufacturer. All multiwell plates were measured with the lid on (a prerequisite for some studies, e.g. when working with axenic cultures). The measurements were done using the following settings: measuring intensity 5, frequency 1 and gain 2. Both F_0 and F_m were measured for every well. A single F_0 and F_m measurement per well was

obtained by averaging the values obtained for all pixels within the well area (area of interest). The F_m values were corrected for a decrease in measuring light intensity during a saturation pulse caused by the heating of the LEDs, according to the manufacturer's instructions. The maximum quantum efficiency of PSII, F_v/F_m, was calculated as $(F_m-F_0)/F_m$ (Consalvey et al. 2005).

Microscopy and automated cell counts

Three pictures (691.2µm x 518.4 µm) were taken of different, randomly chosen locations in each well using a DS-Fi2 Camera (Nikon, Netherlands), mounted on an Axiovert 135 inverted microscope (20x magnification; Carl Zeiss AG, Oberkochen, Germany). Cells were automatically counted using the Cell Counter plugin (De Vos, 2001) in ImageJ (Abràmoff et al. 2004). A size threshold of 32 and 55 pixels was set for *C. closterium* and *S. robusta* cultures respectively. Counts for each well were obtained by averaging the number of cells counted in the three pictures. In addition we noted for each well whether particles (dead cells, silica crystals, extracellular polymeric substances as observed microscopically) were present. The average biovolume of a strain was calculated according to Hillebrand et al. (1999) on approximately 20 cells per culture.

HPLC

After PAM measurements and imaging for counts, Chl *a* concentration was determined in a selection of wells representing the range in biomass among all cultures used in this study. For harvesting, the cultures were resuspended by scraping and pipetting after which they were collected in 2 ml Eppendorf tubes (Eppendorf, Germany). The supernatant was removed by centrifugation (2000 RCF, 10min, 12°C) and the pellet stored at -20°C. Chl *a* was quantified using HPLC (High-performance liquid chromatography) as described in Van Heukelem and Thomas (2001) with the additional step of 30s of bead beating (0.25-0.5 mm beads, Roth) to break open the cells before sonication.

Multiwell plate induced artefacts

To quantify the importance of the position of a well in the plate, the distance from the centre of each well to the centre of the plate was measured with a 0.05 cm precision. To estimate the effect of fluorescence originating from neighbouring cells on the obtained fluorescence reading (crossover fluorescence), the total volume of algal culture added to the wells (to make the dilution series) above, below, to the left and to the right of a measured well was summed.

Statistics

For each treatment we obtained F_0, F_m and F_v/F_m before (indicated with a ^{light} indexation) and after dark adaptation (indicated with a ^{dark} indexation), number of cells (cell counts) and Chl *a* content per well. Pearson correlations were calculated between the PAM and the other proxies for all available wells, but also for only the wells with an F_0^{dark} value below 0.075, and wells with an F_0^{dark} value above 0.075 to evaluate the correlation strength between high and low fluorescence readings.

Simple linear models were constructed with both cell count and Chl *a* as a function of the dark adapted F_0. In order to obtain normality of the residuals and an independent scatter around zero, both the independent (F_0) and dependent (Chl *a*; cell counts) variables were log transformed, which resulted in models of the form ln(Chl a) $=\alpha \times \ln(F_0)+\beta+\varepsilon$ and ln(cell count) $=\alpha \times \ln(F_0)+\beta+\varepsilon$ with α the slope, β the y axis intercept and ε the residual (error). Models were expanded through stepwise forward selection (Neter et al. 1996) with the following variables: species, growth phase (stationary or exponential), biovolume, F_v/F_m, the distance (in cm) from the centre of the multiwell plate to the centre of the well, presence of particles (yes or no) and the sum of the algal concentrations in the neighbouring wells. Terms were added (based on their significance) until no additional significant terms were left or until the variance inflation factors exceeded 10 (which suggests multicollinearity).

To assess the impact of potential multiwell plate artefacts (distance from each well to the centre of the plate, as well as fluorescence cross-talk from diatoms in the neighbouring wells) on the obtained PAM-based biomass proxies, we constructed a new model in which we expressed the PAM-based fluorescence parameters (F_0^{dark}, F_0^{light}, F_m^{light}, F_0^{dark}) as a function of the volume of diatom culture added to the neighbouring wells (to make the dilution series, ranging from 0 to 1.5 ml). As cell abundances differed between the different diatom stock cultures used, we corrected for the different cultures by including diatom culture as a random effect in the model (mixed effects model with stock culture as random effect; R package NLME 3.1-131; Supp. Fig. 2; Zuur et al. 2009). Both the distance from each well to the centre of the plate and diatom concentrations in the neighbouring wells, and all possible interactions between these terms, were included in the mixed effects model. The marginal R^2 (proportion of variance explained by non-random effects alone) was calculated using the MuMIn package (1.15.6). All modelling, graph construction and additional calculations were performed in R (3.4.1).

Results

In total, 231 wells (116 *C. closterium* and 115 *S. robusta* cultures) were measured with the PAM imaging system and used for cell counts. Eighty wells spanning the different treatments and concentrations used in this study were also selected for Chl *a* quantification. The diatom cells did not cluster or overlay each other in the wells and it is therefore safe to state that masking of the fluorescence signal from underlying cells did not occur.

Cell counts and Chl *a* concentration increased more or less linearly with increasing fluorescence-based biomass proxies. However, the best linear relation was found between the fluorescence-based and the other biomass estimators when both were log-transformed (Fig. 2, Supp. Fig. 3; Table 1). Linear models of cell counts and Chl *a* concentration in function of the fluorescence-based biomass estimation were constructed using F_0^{dark} as this is the most commonly used algal biomass proxy (Perkins et al. 2010). Even with F_0^{dark} as the only dependent variable, models were highly significant ($p<0.001$; Supp. Table 1). The fit of F_0^{dark} in function of Chl *a* (adj.

R^2=0.89) was better than that in function of cell counts (adj. R^2=0.78). After the stepwise forward selection procedure, the models (Supp. Table 1) for both Chl *a* (Supp. Fig. 4) and cell counts (Supp. Fig. 5) also included diatom species, growth phase and F_v/F_m. The cell count model additionally included the presence of particles, biovolume, the interaction between diatom species and growth phase, and the interaction between particles and F_v/F_m. The estimate (principal slope) for the increase of F_0^{dark} with increasing Chl *a* or cell counts was about 1 in both cases. Since the biomass proxies were log-transformed, this indicates that their relationship was quasi linear when the above-mentioned factors (species, growth phase, F_v/F_m and particles and biovolume) are also taken into consideration.

Table 1: Cell counts and Chl *a* concentrations in function of F_0^{dark}. The linear models were obtained through forward stepwise selection using the natural log transformed biomass proxies. The effects sizes, their standard error (SE) and the significance of the separate terms are shown.

Chl a = F_0^{dark}+ growth phase + F_v/F_m^{dark} + diatom species			
(df=76; F=1148; p<0.0001; Adj. R^2=0.983)			
	estimate	SE	p-value
F_0^{dark}	1.08	0.03	<0.0001
growth phase	-0.79	0.1	<0.0001
F_v/F_m^{dark}	4.09	0.24	<0.0001
diatom species	-0.17	0.7	0.03
cell counts = intercept + F_0^{dark}+ growth phase+ diatom species + particles + F_v/F_m^{dark} + biovolume + diatom species x growth phase + particles x F_v/F_m^{dark}			
(df=216; F=316.5; p<0.0001; Adj. R^2=0.919)			
	estimate	SE	p-value
intercept	13.97	0.35	
F_0^{dark}	1.01	0.07	<0.0001
growth phase	-1.16	0.15	<0.0001
F_v/F_m^{dark}	4.26	0.53	<0.0001
diatom species	-1.26	0.16	<0.0001
particles	1.5	0.19	<0.0001
biovolume	-0.002	0.0004	0.0002
diatom species x growth phase	0.75	0.19	<0.0001
particles x F_v/F_m^{dark}	-3.69	0.63	<0.0001

The fluorescence parameters F_0^{light} and F_0^{dark} (obtained before and after 15 min dark adaptation) were strongly correlated (r=0.985). F_0^{light} and F_0^{dark} differed most notably for stationary *S. robusta* in which F_0^{dark} was higher than F_0^{light} (Supp. Fig 6). The differences between F_m^{light} and F_m^{dark} were most pronounced for stationary cultures as well: *C. closterium* had higher and *S. robusta* lower F_m^{dark} values compared to their

respective F_m^{light} values. Overall, the correlation between F_m^{light} and F_m^{dark} was high (r=0.995). Substituting the dark adapted fluorescence parameters with the non-dark adapted ones in the linear models (see above) changed little to the regressions (Supp. Table 1). The same was true when substituting F_0 for F_m in the model.

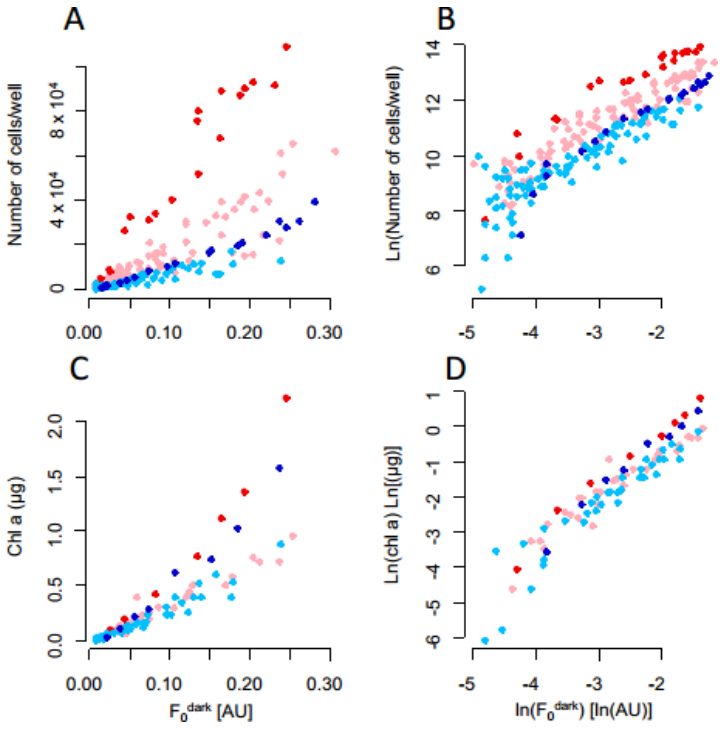

Figure 2: Logarithmic transformation of the data improves linear fit and reduces differences between culture characteristics. The number of cells (A-B) and the chlorophyll concentrations (C-D) per well shown in function of F_0^{dark} before (A&C) and after (B&D) transforming all variables by taking their natural logarithm. Exponential C. closterium cultures in pink and the stationary ones in red, whilst exponential S. robusta cultures are in light blue and the stationary ones in dark blue.

F_0 correlated better than F_m with the counts and Chl *a* concentrations (Supp. Table 2). Only for the lower fluorescence readings (F_0^{dark}<0.075) F_m tended to correlate better with Chl *a* and cell counts than F_0 (Supp. Table 2). At the lowest cell concentration ranges (F_0^{dark}<0.015) the F_m of the wells decreased disproportionally (Supp. Fig. 7) compared to their respective F_0 values. The discrepancy between F_m compared to their respective F_0 values in the lower concentration ranges lead to a loss of variable fluorescence (Fig. 3) in the most dilute cultures. In the higher cell density range (F_0^{dark} > 0.075), this was not the case as all F_v/F_m measurements on the same stock cultures were highly similar. F_v/F_m of cultures in the stationary phase was always lower than in exponentially growing cultures of the same species.

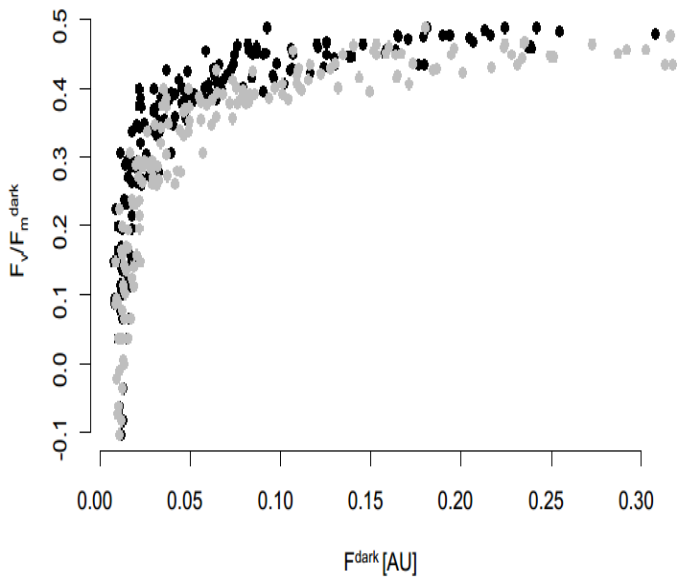

Figure 3: a strong underestimation of the F_v/F_m for lower cell densities. The maximum quantum yield of PSII (F_v/F_m) in function of the dark-adapted fluorescence (F_0^{dark} in black and the corrected F_m^{dark} in grey) for the exponentially growing cultures. Although all cultures shown here were equally healthy, the F_v/F_m ratio was lower below an F^{dark} of 0.075 as a result of the discrepancy between F_m and F_0 at lower algal densities.

In the mixed effects models we expressed the PAM-based biomass proxies in function of the volume of stock culture added. The amount of diatom culture fitted best to the non-dark adapted parameters (Supp. Table 1), although differences were very minor. The highest correlation with the volume of stock culture was also found with F_0^{light} (r=0.86), although differences were negligible (correlation with F_m^{dark}, F_m^{light}, F_0^{dark} is 0.85). The mixed effects model allowed testing the impact of potential artefacts. The volume of diatom culture present in the neighbouring wells (above, below, left and right of the well of interest) had a significant effect ($0.02 \leq p < 0.05$) on the fluorescence values when there was less culture in the well under consideration. The distance from the well to the centre of the plate also tended to be significant ($0.01 \leq p \leq 0.05$) as an interaction effect with both the amount of culture in the well under consideration and in the neighbouring wells. Although significant, the magnitude of this interaction term was small.

Discussion

In this study we investigated the reliability of PAM fluorescence imaging for high-throughput monitoring of growth in benthic diatoms by comparing PAM-based algal biomass proxies with other biomass proxies commonly used to estimate algal growth (chl *a*, cell counts). We evaluated the effect of growth phase (exponential vs stationary) and cell size on the relationship of the PAM based proxies with cell counts and Chl *a* concentrations for two different benthic diatom species over a range of culture densities.

Both F_0^{dark} (measured after a 15 minute dark adaptation period) and F_0^{light} (measured immediately after exposure to growth light conditions) performed equally well as biomass proxies in exponentially growing cultures, as was the case for dark and non-dark adapted F_m. In general, F_m proved to be a reliable biomass proxy for most

conditions used in this study. As maximum fluorescence levels are higher, it could be reasoned that F_m (in comparison with F_0) could have a better signal to noise ratio in diluted cultures. However, deviations from linearity were observed in the F_0-F_m relationship at the lower densities (F_0^{dark}<0.075) and since F_0 showed overall to be a more reliable biomass proxy, we advise to use F_0 for more consistent biomass estimations. The underestimation of F_m at the lower densities, probably an artefact created by the heating of the LEDs during the saturating pulse, led to a decline in variable fluorescence. Therefore, F_v/F_m cannot be reliably estimated at low densities. Nonetheless, at sufficiently high densities, PAM fluorescence imaging has the benefit over other quantification methods in that it can simultaneously provide information on the biomass and the photophysiological status of the cell culture.

A strong effect of growth phase on the PAM measurements was observed, so special care should be taken when cultures approach stationary phase. In addition, the effect of light history (dark adaptation or not) on fluorescence parameter determination was more pronounced in the stationary cultures. This effect differed between species: stationary *C. closterium* had higher, and *S. robusta* lower, F_m^{dark} values compared to their respective F_m^{light} values. This can most likely be attributed to species-specific physiological responses to nutrient depletion (potential mixotrophy in *C. closterium,* Vanelslander et al. 2009). To prevent effects from light exposure during stationary phase, cultures may require special attention in order to obtain stable fluorescence values: adaptation to low light conditions for non-photochemical quenching (NPQ) reversal and a maximal oxidation of Q_A may be necessary (Jesus et al. 2006). Since the cultures were maintained in low light conditions, dark- or low light adaptation could still be required for exponentially growing cultures growing under moderate or high light conditions.

Smaller cells (with a lower biovolume) had, independent of species, less fluorescence per cell. This was not surprising considering that cell size dictates some properties (such as Chl *a*/cell) of the cells which could modulate their photosynthetic performance (Mercado et al. 2004). It therefore could be necessary to re-evaluate the relationship between the number of cells and PAM-based fluorescence for long-term monitoring of growth when a high precision is required. Alternatively, cell size reduction resulting from long-term culturing of diatom strains could be prevented by cryopreserving the cells (Stock et al. 2018).

All wells in a 24-well plate could adequately be measured using PAM imaging. A drawback is the inhomogeneity in measuring light intensity (Schreiber et al. 2007; Vieira et al. 2013) and fluorescence cross-over from neighbouring wells. While inhomogeneity of measuring light intensity can be corrected for, we observed a significant but small underestimation of the fluorescence measured near the edge of the 24-well plates. Such edge effects could be avoided by not using the outer rows of the well plate, as recommended by Schreiber et al. (2007).

The effect of fluorescence originating from surrounding wells on the well of interest was nearly negligible. In extreme situations where fluorescence from surrounding wells is

much higher than in the well of interest, the use of black well plates could improve results by blocking fluorescence originating from the surrounding wells. Alternatively, replicate randomization across a single well plate or grouping species with similar growth characteristics per well plate could diminish the effects of fluorescence from neighbouring wells.

Below, we formulate some recommendations for the use of Imaging-PAM fluorometry as a tool to monitor the growth of benthic diatoms cultures.

For rapid biomass quantification of non-stressed, exponentially growing diatom cultures, dark adaptation can be omitted and both F_0 and F_m can be used as reliable biomass estimates (Perkins et al. 2002, 2010). Extra caution is advised when using PAM fluorescence imaging to monitor growth of very dense cultures or using species that tend to form clusters. A shading effect (cells on top masking the fluorescence signal of the underlying cells) may occur, leading to an underestimation of the fluorescence (Serôdio 2004; Perkins et al. 2010).

A decline in F_v/F_m when cells are stressed (Forster and Martin-Jézéquel 2005; Bender et al. 2014) could be used as an indication that growth estimation through PAM fluorometry is no longer reliable. Robustness of the PAM measurements under these condition can be increased by relying on the dark adapted F_0, which proved to be the most stable fluorescence-based biomass proxy in this study. In addition, the relationship between the PAM-based algal biomass proxies with other biomass proxies was most robust when both were log-transformed. When expressing PAM-based proxies in function of alternative ones over a large range of conditions, a log transformation of the data will most likely improve linearity between proxies.

If stress on the cells is caused by the use of compounds such as herbicides or high light treatments, which directly impact the condition of the photosynthetic apparatus (Dorigo and Leboulanger 2001), PAM-based algal biomass proxies are unreliable. Under these conditions the use of PAM imaging fluorometry should be limited to the short-term imaging of PSII efficiency as described by Schreiber et al. (2007).

Finally, if PAM fluorescence imaging is used to monitor growth in sediment cores and in mesocosms containing substrate, special attention should be paid to an underestimation of the biomass due to vertical migration into the sediment (Honeywill et al. 2002; Jesus et al. 2006). Furthermore, as illustrated by the differences observed between species in this study, the taxonomic composition of natural communities can alter the Chl *a*/F_0 relationship.

In conclusion, PAM fluorescence imaging is a useful technique for high-throughput growth monitoring of benthic diatoms. Fluorescence remains a relative value for biomass estimation and its relation to other biomass proxies should be re-evaluated for use with different species and as diatoms decrease in cell size. Extra caution is advised when applying it under experimental conditions which can affect the photophysiology of the cells. PAM fluorescence is particularly suited for quantifying growth during the exponential growth phase of non-clustering cells at low to

intermediate densities. Dark adapted F_0 seems to be the most stable fluorescence-based biomass proxy, when a complete growth curve (not only the exponential part) needs to be monitored. During high-throughput application of this technique, the relative position of individual wells as well as cell size reduction in particular should be controlled to prevent biases.

Acknowledgements

The research leading to results presented in this publication was carried out with infrastructure funded by EMBRC Belgium - FWO project GOH3817N. This research was also supported under the BIO-Tide project, funded through the 2015-2016 BiodivERsA COFUND call for research proposals, with the national funders BelSPO, FWO, ANR and SNSF. WS was funded by the Fund for Scientific Research – Flanders (FWO-Flanders, Belgium). The BCCM/DCG diatom culture collection is acknowledged for providing us with the appropriate diatom strains for this study. The two anonymous reviewers are acknowledged for their constructive comments on the earlier version of this manuscript

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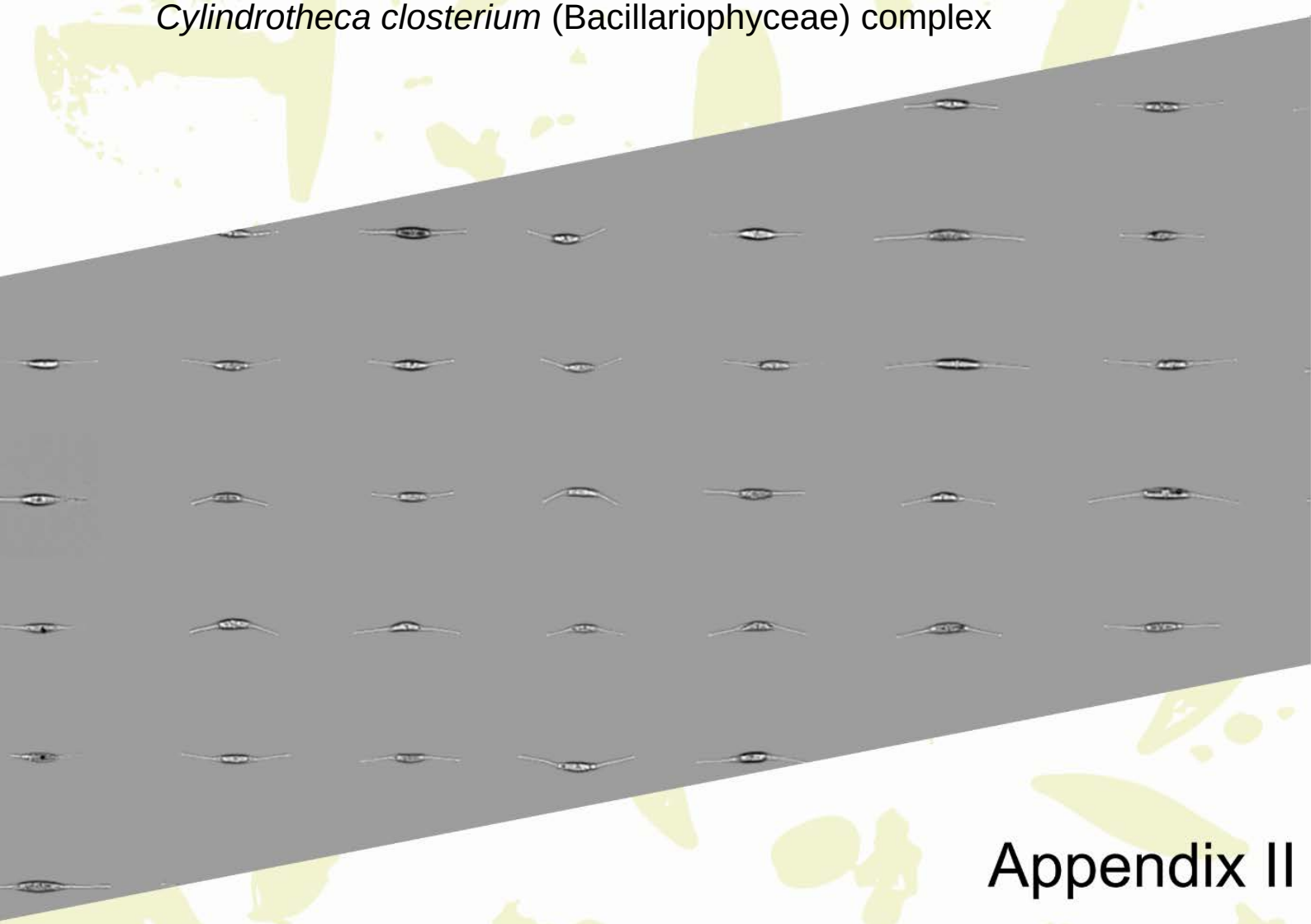
Supplementary material

See: https://www.sciencedirect.com/science/article/pii/S0022098118303459

Appendix II: Thermal niche differentiation in the benthic diatom *Cylindrotheca closterium* (Bacillariophyceae) complex



Appendix II

Thermal niche differentiation in the benthic diatom *Cylindrotheca closterium* (Bacillariophyceae) complex

Published as: Stock, W., Vanelslander, B., Rüdiger, F., Sabbe, K., Vyverman, W., & Karsten, U. (2019). Thermal niche differentiation in the benthic diatom *Cylindrotheca closterium* (Bacillariophyceae) complex. *Frontiers in Microbiology,* 10, 1395.

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Abstract

Coastal waters are expected to undergo severe warming in the coming decades. Very little is known about how diatoms, the dominant primary producers in these habitats, will cope with these changes. We investigated the thermal niche of *Cylindrotheca closterium,* a wide-spread, benthic marine diatom using 24 strains collected over a wide latitudinal gradient. A multi-marker phylogeny in combination with a species delimitation approach shows that *C. closterium* represents a (pseudo)cryptic species complex, and this is reflected in distinct growth response patterns in terms of optimum growth temperature, maximum growth rate and thermal niche width. Strains from the same clade displayed a similar thermal response, suggesting niche conservation between closely related strains. Due to their lower maximum growth rate and smaller thermal niche width, we expect the polar species to be particularly sensitive to warming, and, in the absence of adaptation, to be replaced with species from lower latitudes.

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Introduction

Coastal waters have warmed during the last decades, and are predicted to continue to warm until the end of this century and beyond, potentially by as much as 2 to 3°C depending on the geographical region (IPCC 2013; Schleussner et al 2015). The effects are even more severe for benthic species since sediment surface temperature changes occur most intensively due to strong attenuation of solar radiation (Harrison 1985; Jörgensen and Des Marais 1986).

Evidence is accumulating that rising temperature affects the performance of coastal species in many regions worldwide. Coral reefs are known to be particularly threatened by global warming (Hughes et al. 2017) since the coral-algal symbiosis is living at its upper thermal limit, and temperature anomalies of just 1–2°C above mean local summer maxima are sufficient to cause massive coral bleaching leading to high mortality (Lesser 2011). Also in temperate regions, warming coastal waters affect temperature-sensitive species such as the brown alga *Fucus vesiculosus* in the Baltic and the North Sea (e.g. Graiff et al. 2015). Coastal microalgae on the other hand might be more resistant to global warming (Woefel et al. 2014a; Woefel et al. 2014b). To accurately predict the effects of coastal warming, a better understanding of how temperature and temperature variations will affect coastal marine organisms is required.

Diatoms are a large and often dominant constituent of the coastal microalgal community (Malviya et al. 2016; Underwood and Kromkamp, 1999). Diatoms exist as benthic and pelagic forms and are regarded as one of the largest and ecologically most successful groups of microorganisms on Earth. They are the most diverse group of marine phytoplankton (Armbrust 2009). Apart from dominating intertidal mudflats and shallow water coastal zones, diatoms are at the base of the coastal trophic food webs (Cahoon 1999, Underwood and Kromkamp 1999).

A recent modelling study on *Fragilariopsis kerguelensis*, a dominant diatom species throughout the Antarctic Circumpolar Current and one of the main drivers of the biological silicate pump, indicates that ocean warming might indeed affect the biogeography of this taxon (Pinkernell and Beszteri 2014). Consequently, rising ocean temperature has the potential to alter the composition and the productivity of marine diatom communities, thereby affecting global biogeochemical cycles (Thomas et al. 2012). Predicting the effects of future ocean warming on biogeochemical cycles of carbon, nitrogen, phosphorus, and silicate depends on understanding how existing global temperature variation affects the important marine primary producers (Laufkötter et al. 2015; Roxy et al. 2016).

Although the decisive ecological roles of diatoms are broadly recognized, knowledge about their biodiversity, geographical distribution and possible endemism on different spatial and temporal scales remains limited, particularly for benthic forms. Morphology-based studies have led to the assumption that many marine diatoms are cosmopolitan and ubiquitous (Cermeño and Falkowski 2009; Rad-Menéndez et al. 2015), but detailed molecular studies revealed that many of these alleged cosmopolitan diatoms actually consist of several morphologically identical (cryptic) or almost identical (pseudocryptic) species (Casteleyn et al. 2010; Degerlund et al. 2012; Kooistra et al.

2008). This raises the question whether the supposed ecological plasticity of many cosmopolitan species in fact reflects phenotypic differences between geographically restricted cryptic species. The recognition of cryptic diversity might even explain apparently meaningless patterns in the biology or biogeography of species (Amato et al. 2007). Particularly the importance of geography (Casteleyn et al. 2010) and environment (Kooistra et al. 2008) on the structuring of diatom communities requires further investigation when we want to correctly predict the effects of global change (Usinowicz and Levine 2018).

In addition to the ecological plasticity of diatoms, the evolutionary plasticity of niche characteristics, such as an optimal temperature range, will also be important in predicting species responses to ocean warming (Chivers et al., 2017). The retention of niche characteristics over generations, i.e. niche conservatism, might constrain adaptation in a rapidly changing environment (Pyron et al. 2015). For species living closely to their upper thermal limit and with limited dispersal possibilities, niche conservatism may result in local extinctions (Deutsch et al. 2008; Soininen et al. 2018). Based on field observations, diatoms are thought to not show high niche conservatism (Chivers et al., 2017; Soininen et al. 2018). However, cryptic diversity might be obscuring patterns.

In the present study, *Cylindrotheca closterium* (Ehrenberg) Reimann and Lewin 1964 was chosen as an ecologically important cosmopolitan benthic diatom species. *C. closterium* is widely distributed in high and low latitude marine to brackish water regions where this species can reach high densities (De Brouwer et al. 2005; Najdek et al 2005) and also occurs inside sea-ice (von Quillfeldt et al. 2003). An internet search with the Ocean Biogeographic Information System (OBIS; http://www.iobis.org; accessed on August 6th 2018) resulted in over 30,000 records for this taxon worldwide, indicating a cosmopolitan distribution. However, whether all these records indeed represent the same taxon is unclear since detailed molecular-taxonomical data for most of the samples are missing. Nevertheless, *C. closterium* has been widely used as a diatom model system to study diatom ecophysiology, including the production and function of extracellular polymeric substances (De Brouwer et al. 2005; Pletikapić et al. 2011), movement (Apoya-Horton et al. 2006; Araújo et al. 2013) and anti-oxidative defence (Rijstenbil 2005).

The main goal of this study was to comprehensively investigate the phylogenetic position and origin of 24 *C. closterium* strains in relation to their thermal response. The *C. closterium* strains were collected from tropical, temperate and polar coastal regions and we thus expected to observe pronounced differences in their thermal growth responses. We further anticipated a phylogenetic signal to be present in their thermal response, allowing us to infer a model on their temperature niche evolution and predicting the future impact of global change on the biogeography of *C. closterium*.

Material and methods

Isolation and culturing

For the present study, 24 strains morphologically corresponding to *Cylindrotheca closterium* s.l. were used (Fig. 1).

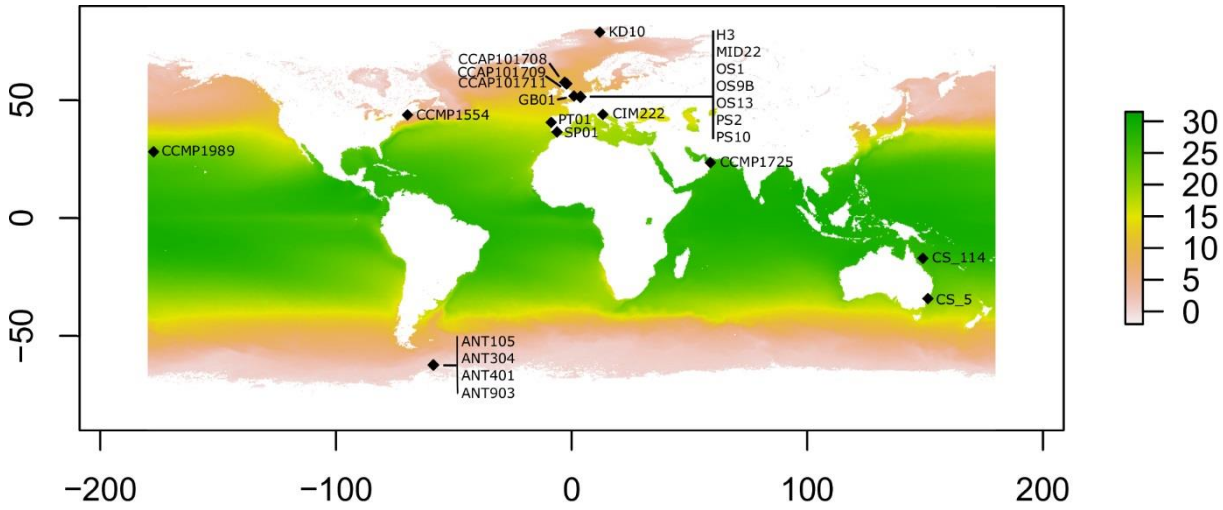

Figure 1: Geographic distribution of the *Cylindrotheca closterium* strains used in this study. Sampling locations with the respective strain names are shown. The average yearly sea surface temperature (in °C) between 2002 and 2018 is indicated by colour.

Details on strain number, isolator, biogeographic origin, climatic zone and geographic location (latitude/longitude) are given in Table 1. Twelve strains were newly isolated from marine and brackish sediment and plankton samples. The strains were isolated from sediments applying the lens tissue technique (Eaton and Moss 1966), in which migratory behavior was used to collect benthic diatoms 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 h of incubation at low light. From this migrated cell population monoclonal cultures of *C. closterium* were established by isolating single cells using a micropipette followed by subsequent culturing in filtered (0.2 µm) seawater (salinity: 33 S_P) enriched with f/2 nutrients (Guillard 1975). The other 12 strains of *C. closterium* were obtained from the National Center for Marine Algae and Microbiota (NCMA), U.S.; 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. Serôdio, I. Moreno Garrido, J. Taylor and M. Pfannkuchen, respectively.

All stock cultures, except the polar strains, were kept in 24-well plates (Greiner Bio-One, Frickenhausen, Germany) at 18 ± 0.3°C with a 16:8 h light: dark period and 50 µmol photons $m^{-2}s^{-1}$ provided by cool-white fluorescent tubes (Philips TLD 18W, Philips Ltd. Eindhoven, The Netherlands) in the weeks prior to the experiment. Originally, the CCAP were maintained at 15°C, CS-114 at 25°C, the (sub)tropical CCMP strains at 24°C and the temperate CCMP strain at 14°C. The polar strains ANT105, ANT304, ANT401, ANT903 and KD10 were isolated and maintained at 6°C, under identical light conditions. All cultures were transferred every 2 weeks to fresh medium.

Table 1: List of *Cylindrotheca closterium* strains studied. Given are strain number, isolator, biogeographic origin, climatic zone, geographic location, coordinates and the yearly average sea surface temperature (SST) of that location Strains are ordered according their biogeography from low to high latitudes.

Strain number	*Isolator*	*Date*	*Biogeographic origin*	*Climate zone*	*Location*	*Yearly average SST (°C)*
CS-114	J.L. Stauber	1980	Coral Sea, Australia	Tropical	17° S, 149° E	26.5
CCMP1725	J. Stirn	1995	Gulf of Oman, Arabian Sea	Tropical	23° 34’ N, 58° 51’ E	27.3
CCMP1989	N. Rolde	1997	Midway Islands, USA	subtropical	28° 12’ N, 177° 21’ W	23.5
CS-5	M. Wotten	1962	Port Hacking, Australia	subtropical	34° 04’ S, 151° 08’ E	19.3
SP01	I.Moreno-Garrido	2000	Puerto Real, Spain	subtropical	36° 36’ N, 6° 12’ W	19.1
PT01	J. Serôdio	2004	Rio de Aveiro, Portugal	subtropical	40° 39’ N, 8° 40’ W	15.0
CIM222	M. Pfannkuchen	2009	Adriatic Sea, Croatia	subtropical	44° 02’ N, 13° 14’ E	18.1
CCMP1554	D. Jacobson	1993	Boothbay Harbor, Maine, USA	Temperate	43°50’N, 69° 38’ W	9.7
PS10	B. Vanelslander	2008	Western Scheldt, The Netherlands	Temperate	51°21' N, 3°43' E	11.9
PS2	B. Vanelslander	2008	Western Scheldt, The Netherlands	Temperate	51°21' N, 3°43' E	11.9
OS13	B. Vanelslander	2007	Eastern Scheldt, The Netherlands	Temperate	51°32'N, 3°44' E	12.9
OS9B	B. Vanelslander	2007	Eastern Scheldt, The Netherlands	Temperate	51°32'N, 3°44' E	12.9
OS1	B. Vanelslander	2007	Eastern Scheldt, The Netherlands	Temperate	51°32'N, 3°44' E	12.9
MID22	B. Vanelslander	2007	Veerse Meer, The Netherlands	Temperate	51°33'N, 3°47' E	11.8
H3	B. Vanelslander	2007	Western Scheldt, The Netherlands	Temperate	51°21' N, 3°43' E	11.9
GB01	J. Taylor	2008	Colne, UK	Temperate	51° 50’ N, 0° 59’ E	12.1
CCAP101711	E. Bresnan	2004	Stonehaven, Scotland, UK	Temperate	56° 58’ N, 2° 12’ W	9.3
CCAP101709	E. Bresnan	2004	Cove Bay, Scotland, UK	Temperate	57° 06’ N, 2° 04’ W	9.4
CCAP101708	E. Bresnan	2004	Buckie, Scotland, UK	Temperate	57° 40 N, 2° 58’ W	9.6
ANT105	B. Vanelslander	2010	King George, Antarctica	Cold	62°13' S, 58°40' W	0.4
ANT304	B. Vanelslander	2010	King George, Antarctica	Cold	62°13' S, 58°40' W	0.4
ANT401	B. Vanelslander	2010	King George, Antarctica	Cold	62°13' S, 58°40' W	0.4
ANT903	B. Vanelslander	2010	King George, Antarctica	Cold	62°13' S, 58°40' W	0.4
KD10	J. Wölfel	2005	Ny-Ålesund, Spitsbergen. Norway	Cold	78° 55’ N, 11 56' E	1.5

Genetic identity and phylogeny

The evolutionary history of the 25 *Cylindrotheca* strains (24 *C. closterium* and one *C. fusiformis* as outgroup) strains was inferred from a multi-locus DNA dataset. Five DNA regions were selected, which included the nuclear ITS region (consisting of ITS1, 5.8S rRNA gene, and ITS2) and D1/D2 region of the LSU rRNA gene , the chloroplast RuBisCO large subunit gene (*rbcL*) and *psbA* and the mitochondrial gene *cox*1. Cells for DNA extraction were harvested from exponentially growing cultures and pelleted by centrifugation. DNA was extracted using the bead-beating method with phenol extraction and ethanol precipitation according to Zwart (1998). After extraction, DNA

was purified with a Wizard© DNA Clean-up system (Promega). PCR products were obtained using previously published PCR primers and protocols (*rbcL* and ITS: Vanelslander et al. (2009), *cox*1: Evans et al. (2007), LSU: Souffreau et al. (2013), *psbA*: Souffreau et al. (2011). PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The sequencing reactions were performed by cycle sequencing (initial step of 1 min at 96°C, 30 cycles of 10 s at 96°C, 10 s at 50°C and 1 min 15 s at 60°C) using the ABI Prism Big-Dye V 3.1 Terminator Cycle Sequencing kit (Applied Biosystems). The resulting sequencing reaction products were analyzed on a Perkin–Elmer ABI Prism 3100 automated DNA sequencer (Applied Biosystems). All sequences newly generated during this study were deposited in GenBank (accession numbers LSU: MH704526- MH704550; ITS: MH716187- MH716211, *rbcL*: MH807634-MH807658, *psbA*:MH819197- MH819219, *cox*1:MH819220- MH819235).

The sequences were aligned using ClustalW (Mega 7.0.14) under standard settings followed by manual curation and trimming of low-quality regions. The sequences for the different loci were concatenated in sequenceMatrix (1.8). This resulted in an alignment of 4182 nucleotide positions: 575 for *cox*1, 797 for ITS, 525 for LSU, 828 for *psbA* and 1454 for *rbcL*. The concatenated data matrix was incomplete for *cox*1, which was missing for 8 strains and *psbA* which was missing for 2 strains. MrModeltest 3.7 (Nylander, 2004) was used to establish the most appropriate model of DNA evolution (GTR+I+G), which was used in subsequent phylogenetic reconstructions. A maximum likelihood (ML) phylogeny was constructed with RAxML (Stamatakis 2014) under default settings with the *C. fusiformis* strain as the outgroup and the alignment partitioned into the different loci. Additionally, single locus ML trees were constructed in RAxML using identical settings. Bayesian phylogenetic inference (BI) was performed using MrBayes version 3.2.6 (Ronquist and Huelsenbeck 2003). The GTR+I+G model was used in which each protein-coding gene (*cox*1, *psbA* and *rbcL*) was partitioned into three codon positions and LSU and ITS were treated as separate partitions, resulting in a total of 11 partitions. The *C. fusiformis* strain was again set as the outgroup. All parameters were unlinked between partitions. Two independent runs of three heated and one cold Metropolis-coupled Monte-Carlo Markov Chains (MCMC) were run for 30 million generations using default settings and with a relative burn-in set. Runs were sampled every 1,000th generation. Convergence and stationarity of the log-likelihood and parameter values was assessed using Tracer v.1.5 (Rambaut and Drummond 2007) after which a relative burn-in of 25% was used.

For species delimitation, two maximum likelihood phylogenies were constructed based on the *rbcL* and ITS sequences. Using only the unique haplotypes and no outgroup, RAxML was used with the settings identical as those used for constructing the concatenated phylogeny. The Poisson tree processes (PTP) model was used to infer putative species boundaries on these ML trees (Zhang et al. 2013). The bPTP server (Zhang et al. 2013) was used to obtain both ML and BI based PTP search results for both phylogenies.

Growth measurements

The temperature requirements for growth in all 24 strains of *C. closterium* and the *C. fusiformis* strain were assessed by culturing at a range of different temperatures (0.5, 5, 10, 15, 20, 25 and 33°C). Each temperature treatment was replicated four times and all strains were acclimated for 4 weeks to the experimental temperatures before growth was evaluated. Cells for growth experiments were always 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. Growth was monitored by pulse amplitude modulated (PAM) fluorometry (MAXI Imaging PAM fluorometer, Walz, Germany) according to the principal methodological approach of Gustavs et al. (2009). The minimum fluorescence yield F_0 was used as a proxy for biomass (Honeywill et al. 2002). The growth rate was determined during the exponential growth phase (4–5 d) as the slope of the linear regression of log2-transformed F_0 fluorescence versus time for individual cultures. The growth rate averaged over the respective replicates was used for further analyses. All the hereafter mentioned analyses were run in R version 3.4.1. Graphics were constructed using ggplot 2 (version 2.2.1).

The relation between growth rate and temperature was modeled according to Blanchard et al. (1996) using the following function:

$$\mu\,(T) = \mu_{max}\left(\frac{T_{max}-T}{T_{max}-T_{opt}}\right)^{\beta} exp\left[-\beta\left(\frac{T_{max}-T}{T_{max}-T_{opt}}-1\right)\right]$$

μ_{max}: maximum growth rate per day ($\mu\ d^{-1}$)
T: temperature
T_{opt}: optimum temperature for growth
T_{max}: maximum temperature for growth
ß: dimensionless parameter, describing the slope of the growth curve

The parameters of the growth equation were identified by minimization of the ordinary least-squares criterion (minpack.lm -version 1.2; Elzhov et al. 2010). The thermal performance range of each strain, defined as the temperature range at which ≥80% of the predicted maximum growth (performance) can be achieved (Huey and Stevenson 1979), was calculated using the rootSolve package (version 1.7; Soetaert, 2009).

Phylogenetic signal

A maximum likelihood consensus tree was converted to an ultrametric tree using penalized likelihood rate smoothing with a lambda value of 0.1 using the ape package (version 5.1; Sanderson 2002). The correlation between the phylogenetic position of the strains and their predicted thermal optimum was evaluated using Pagel's λ (Pagel 1999) and Blomberg's K (Blomberg et al. 2003). The parameters were estimated and corresponding hypothesis tests were performed using Phylotools (version 0.6; Revell 2012).

The similarities between strain phylogeny and geographical origin were tested. The genetic distances between strains (phylogeny) were calculated as the pair-wise

proportion of nucleotide sites at which the *rbcL* sequences, the largest marker, differ between strains using Mega7 (Kumar et al. 2015). The geographical distances between strains were calculated based on the coordinates provided in Table 1. A mantel test (ade4 version 1.7; Dray and Dufour 2007) was used to test if there was a relationship between the genetic and the geographical distance matrices.

Viability after incubation at extreme temperatures

For a subset of *C. closterium* strains which did not show growth at 0.5°C or 33°C, cell viability was evaluated after 4 weeks incubation at the respective temperature using the fluorescent dye SYTOX-Green. Cells with intact cell membranes were distinguished from those with permeabilized membranes, using the nucleic acid stain SYTOX-Green according the protocol of Veldhuis et al. (2001). SYTOX-Green can only pass through compromised or damaged membranes, and stains the nucleus leading to enhanced fluorescence under blue light excitation. Consequently, viable *C. closterium* cells can be distinguished and quantified from dead cells using epifluorescence microscopy.

Strains CS-114, CS-5, SP01, PT01, CCMP1554, PS10, MID22 and GB01, which did not, or minimally, grew at 0.5°C, were kept for 4 weeks at this temperature. To test their tolerance at higher temperature, strains KD10, GB01, H3, MID22, OS1, OS13, PS2 and SP01, which did not, or minimally, grew at 33°C, were maintained at this enhanced temperature. Media and light conditions were identical to those of the other growth experiments already described above. Media were refreshed every second week.

After 4 weeks of treatment, cells were harvested by gentle centrifugation at 1000 × g for 2 min (Heraeus Labofuge) and the pellets incubated in one drop of diluted SYTOX-Green (Invitrogen) stock solution for 5 min according the manufacturers protocol (see also Veldhuis et al. 2001). The stained cells were counted under an inverse epifluorescence microscope (IX70, Olympus, Hamburg, Germany) at a magnification of 40×. For each of the 3 replicate samples 400 cells were counted as viable or dead.

Sea surface temperatures

The sea surface temperature (SST) was calculated using remote sensing data for the locations from which the strains were isolated. As the locations of interest were generally at the coast, an 8 km buffer around each location was used to prevent the use of data from a mixed ocean/land pixel. The night time skin temperature data, measured at 11 µm band by MODIS-Aqua (NASA OB.DAAC, Greenbelt, MD, USA) was averaged for each season between 2002 and 2018 (the full time window for which these data were available) at a 4 km resolution. The average yearly SST was then calculated by averaging the seasonal SSTs and standard deviation between seasonal SSTs was calculated to represent the seasonal variability in SST at each location. Although these oceanic SSTs do not necessarily best reflect the locally experienced temperatures by the coastal strains (Potts & Swart 1984), they were considered to reliably reflect the general differences between locations. The SSTs were therefore used as a proxy for the in-situ conditions.

Results

Genetic identity and phylogeny

The phylogenetic analyses, using the five different loci, resulted in a well-supported tree (Fig. 2) for the shallow-branching nodes. For deep-branching nodes, support was generally low and appears to have been caused by the topological incongruence of the different markers.

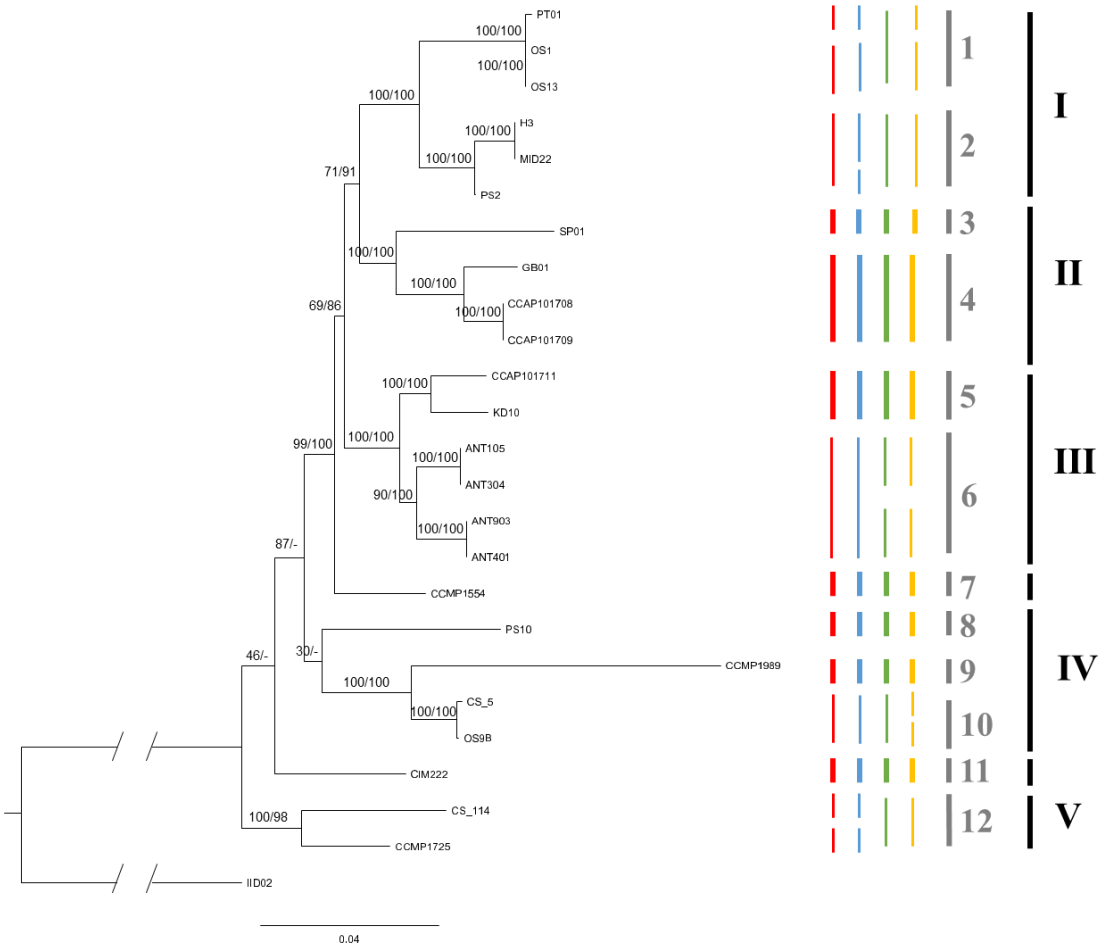

Figure 2. The maximum likelihood consensus tree based on 1000 rapid bootstrap inferences of a concatenated five molecular loci alignment in strains of *Cylindrotheca closterium*. Numbers at nodes are the bootstrap values (ML) and posterior probabilities (BI) respectively, indicating the support for each node; branch lengths represent the expected number of substitutions per site. The truncated part of the outgroup represents an additional 0.22 expected substitutions per site. Clades, indicated by the black bars and roman numerals (see results) were defined based on similar thermal responses. The colored bars represent the putative species based on different PTP models: red (ML search method) and blue (BI search method) are the putative species delineated using the ITS data, whilst green red (ML search method) and yellow (BI search method) are the putative species based on the *rbcL* data. The species delineation bars are in bold if the four predictions are consistent for that species prediction. The grey bars indicate the conservatively delineated putative species based on the four predications. Each species is indicated with an Arabic numeral.

The different PTP models, used to infer putative species boundaries, suggested the presence of multiple species within the *C. closterium* phylogeny (Fig. 2). Based on the ITS phylogeny, 15 and 14 putative species were delineated using the BI and ML search method, respectively. The same approaches resulted in 15 and 13 putative species based on the *rbcL* phylogeny. A conservative consensus estimate, considering only the species delineated by all four methods, resulted in 12 putative species (Fig. 2, grey bars). Many of the delineated species were only represented by a single strain.

Despite the clustering of some strains with the same geographical origin (for instance the Antarctic isolates), there was no significant relation between geographical distances and genetic distances of the strains (Mantel test, p=0.07). This can largely be explained by the high genetic diversity found at some of the locations, particularly at the Scheldt in the Netherlands.

CCMP1989, a strain from an isolated island (Midway Islands, USA) in the Pacific, is quite distinct from the other strains. This is due to the cumulative effect from unique nucleotide differences in several of the loci. Similarly, another strain from the United States (Maine), CCMP1554 forms a distinct lineage on its own. The same is true for CIM222, a strain originating from the Adriatic Sea.

Based on the phylogenetic tree, several clades could be delineated (indicated by roman numerals in Fig. 2) which contain strains that have similar thermal optima (Fig. 3). Each of these clades consists of several putative species. Both Clade I and IV contain temperate strains from the Netherlands (Clade I: OS1, OS13, H3, MID22, PS2; Clade IV: PS10, OS9B; Table 1) and subtropical strains (Clade I: PT01; Clade IV: CCMP1989 & CS_5). Similarly, Clade II includes both temperate strains from the UK (CCAP101708, CCAP101709, GB01) and a subtropical strain from Spain (SP01). Clade III also contains a strain from the UK (CCAP101711) as well as strains from colder regions, Spitsbergen, Norway (KD10) and Antarctica (ANT). Lastly, Clade V contains two tropical strains from the Arabian Sea (CCMP1725) and Australia (CS-114).

Temperature requirements for growth

The investigated *C. closterium* strains showed different growth responses to the applied temperature regimes. None of the isolates grew across the entire temperature range between 0.5 and 33°C (Fig. 3). Temperature requirements for growth generally corresponded closely to the biogeographical origin of strains. With the exception of the fifth delineated species (Fig. 2), all strains of the same species also showed very similar growth responses to temperature.

Most of the subtropical and tropical strains exhibited growth optima above 20°C and upper tolerance levels of 33°C or higher (Fig. 3). In contrast, all polar strains showed optimum growth around 10°C. The temperate strains originated from different habitats between 43°N and 57°N and all grew best around 20°C. Remarkably, for some strains, there was no overlap between thermal performance ranges.

The temperature response of the strains was also compared to their relative phylogenetic position. Two quantitative measures were calculated to express the

phylogenetic signal present in the variation observed in the predicted thermal optima: Pagel's λ (Pagel 1999) and Blomberg's K (Blomberg et al. 2003) Both are significantly different from zero (Pagel's λ =0.943616 with p<0.0001 and Blomberg's K =0.02 with p =0.004), indicating that related strains indeed have the tendency to resemble each other's thermal optimum. Thermal niches thus seem to be, at least, partly conserved within the *C. closterium* complex.

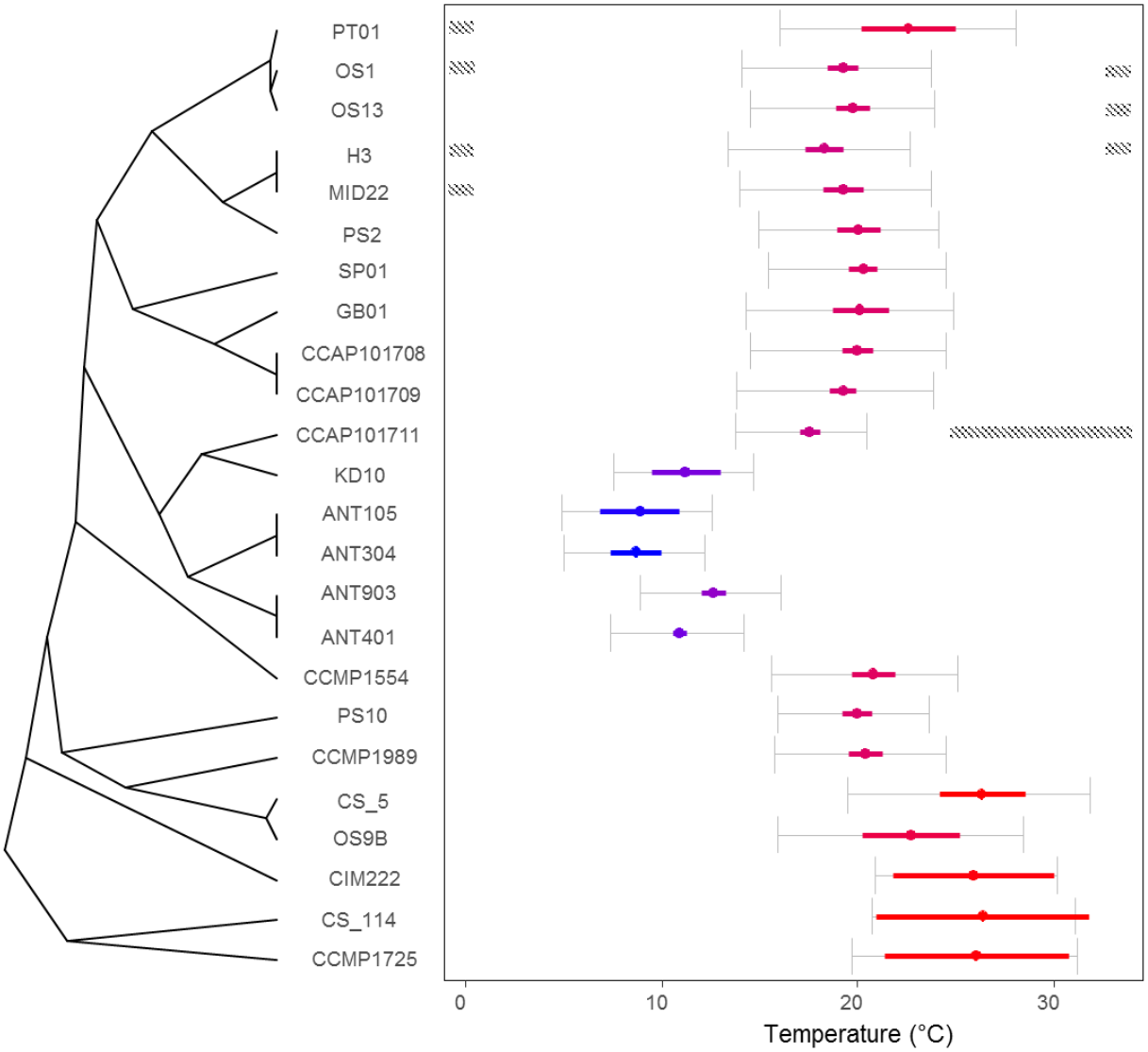

Figure 3: thermal optima of the *Cylindrotheca closterium* strains relates to their phylogenetic position. The cladogram on the left shows the phylogenetic relations between strains and the estimated optimal temperature is shown by the coloured dot (±SE) in the temperature plane on the right. The colour indicates the relative position of the optimal temperature in relation to the other strains (blue colder to red hotter). The grey error bars indicate thermal performance range (the temperature range at which ≥80% of the predicted maximum growth is estimated) of each strain. The dashed bars indicate areas outside the tolerance range, where no growth was detected. Lastly, the squares on both sides of the temperature plane represent the results for the viability assay: a white square indicates that viable cells were detected in the culture four weeks after incubation at 0.5°C (left side) or 33°C (right side), whilst a black square indicates the absence thereof.

For Pagel's λ and Blomberg's K, a value of zero would suggest phylogenetic independence of the thermal response whilst a value of one would indicate that the thermal optimum of species is perfectly distributed along the phylogeny as expected under Brownian motion of trait evolution. Whilst Pagel's λ is close to one, this is not the case for Blomberg's K. Blomberg's K has been shown to be more sensitive to aspects such as the number of strains in the phylogeny (Molina-Venegas & Rodriguez 2017; Münkemüller et al. 2012). Visual inspection confirms the link between thermal optimum and phylogenetic position (Fig. 3) as the strains within clades generally show a similar thermal optimum. There is no clear trend with the sequence of clades as the polar clade is nested within the temperate clades, nor is there a strong link of the thermal optimum with the temperature the strains were maintained at.

The predicted thermal optima correlated closely with the average SST from the location they were isolated from (Pearson correlation coefficient of 0.89; $p<0.0001$, Fig. 4). An increase in average SST of 1 °C was met with an expected increase of 0.6°C in thermal optimum. Similar relations were found when comparing the different seasonal SSTs to the thermal optima (r_{Summer} =0.89, r_{Autumn} =0.90, r_{Winter} =0.75, r_{Spring} =0.88).

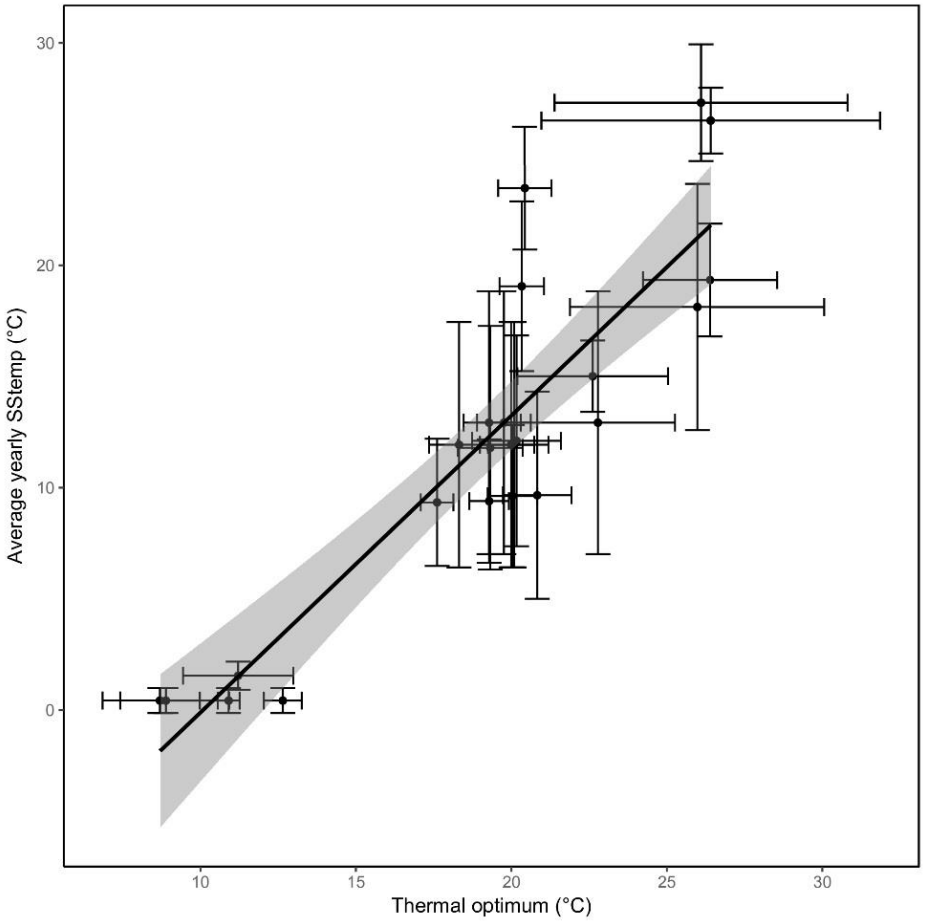

Figure 4: A strong correlation between sea surface temperatures and thermal optima. The predicted optimal temperature (x-axis) correlates very well with the average yearly sea surface temperature from where the strains were isolated (y-axis). The regression through the data points is shown with the confidence interval shaded in grey. The standard deviation of sea surface temperature based on the seasonal variation and the standard error of the predicted optimal temperature, as estimated by the model, are represented as error bars.

The thermal performance range width differed between species (Fig. 3) and was generally smallest for the polar strains. The width increased approximately with 0.26°C for every 1°C increase in optimal growth temperature of the strains ($p<0.0001$; Pearson correlation = 0.78). The width correlated marginally (Pearson correlation coefficient of 0.36; $p = 0.09$) with the standard deviation of the SST from where the strains were originally isolated. A stronger positive correlation was found between the thermal performance range width and the predicted maximum growth rate (Pearson correlation coefficient of 0.69; $p = 0.0002$).

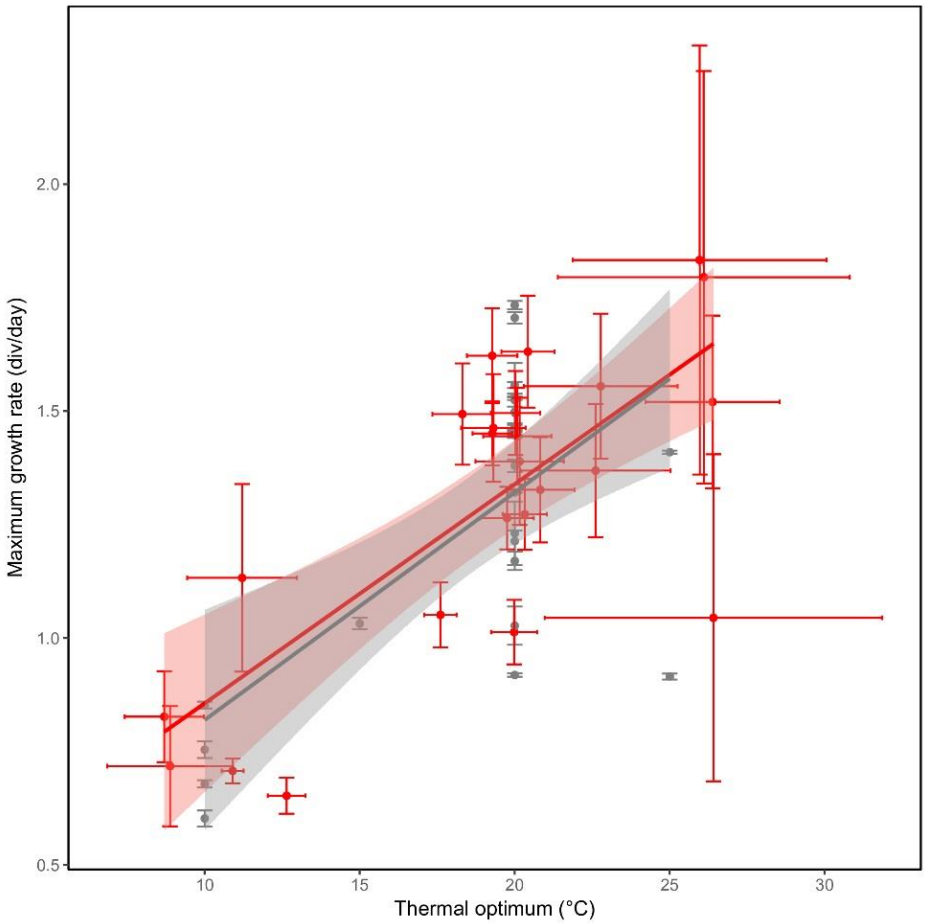

Figure 5: Hotter is better. A higher maximum growth rate in *Cylindrotheca closterium* strains (y-axis) is observed in strains with a higher thermal optimum (x-axis). The grey points represent the experimentally observed maximum growth rate (±SE) and the temperature at which this was observed whilst the red points represent the predicted maximum growth rate with the predict thermal optimum. For both predicted parameters, the standard error is shown as estimated by the model. Linear models were fitted to each of the datasets in the respective colors.

Besides differences in optimum temperature and thermal niche width, maximum growth rate also differed between the investigated *C. closterium* strains. The observed maximum growth rate ranges from to 0.6 to 2 divisions per day. The predicted maximum growth rates, based on the growth equations, had a similar range: from 0.65 to 1.8 divisions per day. The observed and predicted rates correlated strongly (Pearson

correlation =0.97, p<0.0001), confirming the reliability of the model. A strong and significant (p<0.001) positive linear relation was observed between the (predicated) maximum growth rates and the thermal optima of strains (Fig. 5). On average, an increase of 1°C in thermal optima was accompanied with a maximal growth rate increase of 0.05 divisions d^{-1}.

Cell viability under extreme temperatures

Application of the SYTOX-Green stain indicated that survival at 0.5°C was generally better compared to exposure at 33°C in the studied *C. closterium* strains (Fig. 3). Six out of 8 tested strains (SP01, PT01, CCMP1554, PS10, MID22, GB01) exhibited 5-18% viable cells after 4 weeks exposure at 0.5°C, indicating that, although growth could not be measured in these isolates, at least part of the population can survive. In contrast, the tropical strain CS-114 and the subtropical strain CS-5, both originating from Australian waters, did not survive at this low temperature (Fig. 3).

The temperature effect at 33°C was more severe, since only 2 out of 8 selected strains (H3, MID22) showed 2-8% viable cells after 4 weeks incubation. All other tested *C. closterium* strains did not survive this temperature.

Discussion

The phylogenetic analysis based on the five loci resulted in a well-resolved phylogeny which exposed the high genetic variability present within *C. closterium* (Fig. 2). Based on phylogenetic evidence and mating experiments, Li et al. (2007) and Vanormelingen et al. (2013) already suggested, the existence of a cryptic species complex rather than a single *C. closterium* species. The species delineation results, which indicate the presence of up to 15 different cryptic species in this study alone, confirm these suspicions. Detailed taxonomic studies using morphological analyses and reproductive assays in addition to the molecular information will be required to fully resolve this species complex as was done for other species complexes (Amato et al. 2007; De Decker et al. 2018). Furthermore, an extended sampling effort will probably increase the number of extant species and allow to statistically detect biogeographical patterns such as isolation by distance, if they are present (Casteleyn et al. 2010).

The present study indicates different thermal growth optima for the 24 investigated *C. closterium* strains, with closely related strains displaying similar thermal niches. The thermal niche is largely conserved within species and even among closely related species. The strain-specific temperature requirements for growth indicate that the *C. closterium* species complex consists of numerous genotypes which might explain the ecological success of *C. closterium* in geographically widely distributed habitats. Furthermore, the high genetic diversity among the temperate strains and a polar clade consisting of northern and southern hemisphere strains provides evidence for the high dispersal capability of *C. closterium*. The ability of *C. closterium* to disperse easily to the water column (Araújo et al. 2013), probably promotes dispersal in this species complex.

The obtained thermal optima were in accordance with previous studies. Scholz and Liebezeit (2012) investigated a *C. closterium* strain from a German intertidal mud flat of the North Sea and reported a thermal niche width between 10°C and 25°C which corresponds well with the growth response patterns of most of the temperate strains

of the present study. Morris and Kromkamp (2003), however, showed temperature optima for photosynthesis at 30°C in a *C. closterium* isolate from the Ems-Dollard Estuary, The Netherlands. This is much higher compared to the growth optima of the 24 *C. closterium* strains investigated in this study, ranging between 9 and 27°C. Temperature optima for photosynthesis, however, can be higher than those for growth because both physiological processes are not directly coupled in algae (Davison 1991, Eggert and Wiencke 2000). This indicates that temperature effects on a specific physiological process like photosynthesis do not necessarily match the temperature-growth pattern because growth as a more general physiological process integrates all positive and negative influences of temperature on the whole metabolism and hence better reflects physiological activity and viability (Bulthuis 1987). The temperature requirements for growth of the Arctic *C. closterium* strain KD10 confirm the results on other benthic diatoms in this biogeographic region (Karsten et al. 2006). Surprisingly, the 4 Antarctic strains of *C. closterium* exhibited a similar thermal response with a relatively broad growth temperature tolerance compared to typical endemic Antarctic benthic diatoms (Longhi et al. 2003). Hence it is reasonable to assume that they do not carry the same physiological traits as these typical endemics.

Thermal niches were conserved within clades of *C. closterium*, with the majority of the clades growing best around 20°C. It is plausible that there is a stabilizing selection for *C. closterium*, a selection in favour of this intermediate temperature and potentially against extremer temperatures. Under such a scenario, some clades will adapt to thermal extremes, but most will be drawn back towards this more common thermal optimum around 20°C. This is in accordance with the lower, yet significant, Blomberg's K observed in this study (Ackerly, 2009) and the nestedness of cold-water clade III within temperate clades II and IV.

Very little is known about the role of thermal adaptation in speciation (Keller and Seehausen 2012). If temperature does play a major role in ecological speciation, we would expect closely related species to have different thermal niches, and a tight association between cladogenesis and divergence in thermal habitats (Svensson 2012). Such patterns were not observed. Notably, some of the strains belonging to different putative species co-occurred and had a similar thermal niche. This does not rule out the effects of temperature on speciation as we also observed thermal exclusion between several strains: these strains cannot coexist due to their thermal demands. If not responsible for the speciation itself, thermal adaptation might be relevant in the spatial isolation of species.

Compared to the temperate and (sub) tropical strains, the polar strains had a much narrower thermal performance range. The width of the thermal performance range was only weakly linked to the geographical distribution of the strains and seemed to be more dependent on the physiology of the strains. Strains with a higher thermal optimum and growth rate also tended to have a larger thermal performance range. This is in contrary to the *Jack-of-all-trades is a master-of-non* hypothesis (Huey and Hertz 1983), which states that the ability to perform at a broad temperature range can only be achieved at the sacrifice of a high growth rate. In other words, the hypothesis claims there is an expected trade-off between performance breadth and maximal

performance, which was not the case here. The positive correlation between the maximum growth rate and thermal optimum is however in line with the *hotter is better* hypothesis (Huey 1989). This hypothesis states that high temperatures inevitably accelerate biochemical reactions and thus growth. Similar patterns have been observed for other organisms (Knies et al. 2009) and will have important implications for the response of *C. closterium* to climate change. In contrast to Thomas et al. (2012) who predicted tropical microalgae are more sensitive to increasing temperatures due to their optima being closer to the currently experienced temperatures, our results suggest that polar strains are the most vulnerable with respect to global change. The much narrower performance range and slower growth rates of the polar strains will be a large handicap when competing with temperate strains under higher temperatures. However, the thermal tolerance of polar strains might evolve as temperatures gradually increase, allowing polar strains to persist (Schaum et al. 2018). It is worth mentioning that predictions on the effects of global change on coastal microalgae remain largely speculative. Climate change will not result in uniform warming of coastal waters (Liao et al. 2015) nor will its effects be limited to temperature alone. Changes in precipitation and evaporation will act on the local salinity and nutrient concentrations (Scavia et al. 2002), which will in turn affect the capability of the algae to respond to temperature fluctuations (Singh & Singh 2015).

Adaptation to a different temperature regime can be achieved through a combination of different mechanisms. For instance, by increasing the intracellular concentrations of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), a key enzyme for photosynthesis, to compensate for its poor catalytic efficiency at low temperatures (Devos et al. 1998). Other adaptive mechanisms include the evolution of cold or heat shock and antifreeze proteins, the modulation of the key enzyme kinetics, and an alternate composition of the biological membranes through the incorporation of different polyunsaturated fatty acid chains (Morgan-Kiss et al. 2006). It is likely that many of these mechanisms are present in *C. closterium*, which would to explain the broad thermal tolerances that was observed for some *C. closterium* strains, allowing them to survive the high temperature fluctuations typical of most coastal habitats. Whole genome sequencing combined with transcriptomics could provide more insight on the presence and relevance of such thermal adaptations (Mock et al. 2017; Stillman & Armstrong 2015).

Membrane integrity, as an important defence barrier against all environmental influences, is considered as the prerequisite for the cell's survival, and hence it was reported to be the least stress-sensitive trait of microorganisms (Freese et al. 2006). Here we showed that strong cell biological damage occurred in many *C. closterium* strains during 4-weeks exposure at very low, but particularly at very high temperatures (Fig. 3). The SYTOX-Green assays indicate that the thermal niche width for growth can be confirmed by the membrane properties and hence viability, i.e. lower and upper temperatures for growth, are in close proximity to the thresholds for mortality.

Conclusion

In conclusion, we have found a large genetic diversity within *C. closterium* which most likely represents multiple (pseudo)cryptic species. The strains in this species complex

display diverging thermal responses which might explain the global ecological success of *C. closterium*. Due to the narrower thermal niche and lower maximal growth of polar strains, we foresee a potential replacement of cold-adapted species by those originating from warmer areas as water temperatures continue to rise. Additional research on the structure of the cosmopolitan *C. closterium* complex will provide us with the opportunity to better understand their biogeography and, when combined with information on their thermal response, will help to better predict the effects of climate change on these key organisms of shallow water coastal zones.

Acknowledgements

The authors gratefully acknowledge the provision of diatom strains by J. Serôdio, I. Moreno Garrido, J. Taylor and M. Pfannkuchen. The BCCM/DCG culture collection is recognized for their help with the maintenance of the cultures. We are grateful to A. Castagna who aided in obtaining and processing the sea surface temperatures and S. D'Hondt which performed part of the molecular work. E. Pinseel's aid with the species delineation and her comments on the earlier version of the manuscript were greatly appreciated. The work of W. Stock was funded by the Fund for Scientific Research – Flanders (FWO-Flanders, Belgium). Additional funding was provided by Ghent university (grant BOF15/GOA/17). The work on the Spitsbergen isolate has been performed at the Ny-Ålesund International Arctic Environmental Research and Monitoring Facility and under the agreement on scientific cooperation between the Alfred Wegener Institute and the University of Rostock. The authors thank the crew at the AWIPEV-base in Ny-Ålesund and the German dive team (Anita Flohr, Peter Leopold, Max Schwanitz) for assistance in the field, collecting samples and further support, as well as Juliane Müller for maintaining the stock collection of Arctic benthic diatoms at the University of Rostock. We are grateful to the three reviewers whose constructive comments greatly helped to further improve this manuscript. Financial and logistic support was provided by the German Research Council to UK in the frame of the Deutsche Forschungsgemeinschaft (DFG) priority program 1158 "Antarctic Research" (DFG, KA899/12, KA899/15).

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Supplementary material

See: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01395/full#supplementary-material

Acknowledgements

Dankwoord

Today, five years and nine months after starting my PhD, I'm writing this final piece of my dissertation. I could say that it has been a long and difficult road to make it sound more heroic, but in reality it has just been a really nice journey, mostly because of all the people whom have been there along the way. I would therefore like to thank all those people for their support, friendship, the opportunities and time they have given me.

I'll start off with the main responsible of me being here. Koen, I greatly appreciate the freedom you have given me, the trust you have in me and the patience you have to further improve the texts I've written. I'm also thankful to the head of our lab, Wim, for giving me the opportunity to work in his lab and for his scientific insight and advice on the experiments we've done. I wish to extend a big thank you to Sven and Anne, your know-how on the underlying biochemistry and bacterial processes & the attention to detail have been a great asset in my PhD.

Next, I would like to thank all my colleagues & friends at the PAE and Phycology. You bring (rainbow) colours to my days and you are what made these years so memorable. The short talks in between, the lunches, the board game evenings, ice cream breakes all made the working days fly by. To my officemates, Peter, Frederike, Caroline, Jihae, Anja, Alex, Luz, Bayeh and – lest we forget - Lander thank you for the good times and the lovely company. At Lander & Peter, also thank you for your scientific advice and help with the lab equipment. Eveline, thank you for all the good talks, looks like I will have to find someone else to complain with when you are in the States. To the Alfies, Freddy ('verdeelstekker'), Javier ('for the earth!') & Kath('grondboontjiebotter'), it was cool to share our algal-bacterial discoveries and to be able to work on our marine holobionts together. Soria, thank you for cheering up the train rides to Ghent. This will no longer be the case since you have a place in Ghent now (which is likely to have a positive effect on my productivity), but there will still be opportunities for an occasional tea break. At Sam & Andrea, porco dio che divertimento è stato! To all the cynical colleagues, Maxime, Heidi, Bjorn, Kenny, thanks for the black humour & dark jokes. On a similar note, Quinten, thank you for laughing at my lame jokes. To the *C. closterium* team, Darja, Sien,Katerina, thank you for making C CLOS great again! Best of luck to everyone finishing soon (Eveline, Freddy –Dr. Stock²!-, Kath, Eli, Javi) and those tackling difficult challenges in the lab (Lotte, Gust, Luz, Alex,…)

A special thank you to Ilse, Sofie, Tine, Renaat & Olga. I would not have gotten this far without your help, advice and experience: you make the labs run smooth, the sampling campaigns successful and the analyses a walk in the park!

Starting a PhD is difficult, but I didn't have to do it alone. Thank you Clio, Eva, Marleen, Annelien for getting me hooked on marine research and mudflats in particular. I'm grateful to the MarBiol people for the warm welcome: Annick, Guy & Isolde for getting me setup, Lisa, Ee Zin, Pieterjan, Anna-Maria, Tom, Dirk, Bart ,… for the nice atmosphere, the support & the parties. Learning to work with living organisms wasn't as easy as I would have hoped, so thank you Evelyn & Pieter for your teaching on diatom cultivation and thank you Anne, Liesbeth, Evie, Pia, Helen and the LMG people for the help with the bacteria.

Doing research is great, but having someone else to do it for you is better! Thank you to all the students (Raven, Coco, Mieke, Pearl, Evelyn, Giacomo, Laurens, Max, Wannes, Pieterjan, Febe, Frederik) whom I had to honour of supervising. It really has been a pleasure to get to know you all and to share my research with you. I have learned a lot by working with you and I hope that you are doing well.

There are some people I would like to thank for making everything run so smoothly during my PhD. Anja of course, for getting all the paperwork sorted and helping me find my way around the Ugent administration. My laptop has suffered a lot during the last years, but it is still running thanks to Jeroen! I'd like to think that I have been sanitary, but the population of silverfishes (*Ctenolepisma longicaudata*) which inhabited the papers on my desk convinced me otherwise, so thank you Dominique for doing your best to keep it as clean as possible. And, lastly, my (old) running buddies, Thomas, Soria, Quinten, Sien, Darja, Jolien, Céline, An-Sofie, thank you for keeping me energized with our running breaks.

Natuurlijk zou ik ook graag mijn vrienden en familie bedanken. Bedankt Laurent & Katlyn, T, Ben, Wouter, Nelis, Alexia & Jurgen, Dieter, Ine & Levi, Gilles, Julie, Emily en Angelo voor de fijne avonden tussen het schrijven/werken door. Aan mijn (schoon)familie, bedankt voor de oprechte interesse in mijn werk, hopelijk is het na vandaag allemaal wat duidelijker geworden. Jo, Ingrid, Sharon & Yannick, bedankt om mij in de familie op te nemen en sorry voor alle weekends dat ik in Limburg zat om dan alsnog –vrij asociaal- achter mijn laptop te zitten. Tante Marijke en mijn grootouders, dankzij jullie ben ik biologie gaan studeren. Tante en bompa, bedankt om mee op sleeptouw te nemen naar natuurhistorische musea. Opa voor de natuurwandelingen en oma om uren naar me te luisteren als ik weer eens aan het doordraven was over één of ander beest die die dag mijn pad was gekruist. Zus en Roel, bedankt voor jullie steun en om, ondanks jullie drukke agenda's, tijd voor me vrij te maken. Mama & papa, het zijn bewogen jaren geweest, maar desalniettemin kon ik altijd op jullie steun rekenen. Dat ik hier ben geraakt heb ik grotendeels aan jullie te danken, bedankt om in mij te geloven en voor alle kansen die jullie mij gegeven hebben!

Tot slot wil ik nog één iemand bedanken. Bij het begin van mijn doctoraat nog maar net mijn vriendin, in iets wat vooral een lange afstandsrelatie bleek te zijn, en nu toch al meer dan twee jaar mijn vrouw. Je hebt me heel het doctoraat bijgestaan, van bij de beslissing om bij de PAE te beginnen tot aan de voorbereidingen van de receptie straks. Je zette 's ochtends mijn gerief klaar als ik weer eens de klok uit het oog verloren was en zorgde ervoor dat er 's avonds eten klaar stond als ik na een lange dag thuis kwam. Je hebt me geleerd om op tijd mijn werk even aan de kant te leggen, maar het blijft toch een moeilijke oefening om voldoende tijd voor je vrij te maken. Ik blijf eraan werken en met een mooie zomer in het verschiet, hoop ik toch al wat goed te kunnen maken. Tara, mijn vlam uit Limburg/lerares uit West-Vlaanderen, bedankt!

So in the end I'd like to say, That I'm a very thankful man
I tried to make the most of my situations, And enjoy what I had
And I had some regrets, But if I had to do it all again
Well, it's something I'd like to do
Mark Everett. Blinking Lights and Other Revelations. Vagrant. 2005.

Curriculum vitae

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Employment

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Promotors: Prof. Dr. Koen Sabbe, Prof. Dr. Wim Vyverman, Prof. Dr. Ir. Sven Mangelinckx & Prof. Dr. Anne Willems

Education

2011-2013 **Master of Science in Biology** **University of Ghent**

Graduated with greatest honor

Majors: Ecology and Biodiversity

Dissertation: Effects of copepods, diatoms and their interactions on denitrification in marine sediments: an experimental approach

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2008-2011 **Bachelor of Science in Biology** **University of Ghent**

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Dissertation: The seasonal and spatial dynamics of bacterial communities in coastal ecosystems

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2003-2008 **Secondary education** **Royal Atheneum of Kortrijk**

Majors: Mathematics and sciences

Publications

Stock, W., Vanelslander, B., Rüdiger, F., Sabbe, K., Vyverman, W., & Karsten, U. (2019). Thermal niche differentiation in the benthic diatom Cylindrotheca closterium (Bacillariophyceae) complex. *Frontiers in Microbiology*, **10**, 1395.

Koedooder, C., **Stock, W.**, Willems, A., Mangelinckx, S., De Troch, M., Vyverman, W., & Sabbe, K. (2019). Diatom-bacteria interactions modulate the composition and productivity of benthic diatom biofilms. *Frontiers in Microbiology*, **10**, 1255.

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*Jocqué, R., Jocque, M., **Stock, W.**, Naroeun, R. I. N., & Henrard, A. (2019). The new Southeast Asian genus Cambonilla gen. nov.(Zodariidae, Araneae):'bis repetita placent'. European Journal of Taxonomy, 501, 1-24.*

*Dittami, S. M., Arboleda, E., Auguet, J. C., Bigalke, A., Briand, E., Cárdenas, P., ... **Stock, W.**, …&* Not *F. (2019). A community perspective on the concept of marine holobionts: state-of-the-art, challenges, and future directions (No. e27519v1). PeerJ Preprints.*

*Stock, F., Syrpas, M., Graff van Creveld, S., Backx, S., Blommaert, L., Dow, L., **Stock, W.**,... & Vyverman W. (2019). N-acyl homoserine lactone derived tetramic acids impair photosynthesis in Phaeodactylum tricornutum. ACS chemical biology. **14**, 2, 198-203*

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***Stock, W.**, Heylen, K., Sabbe, K., Willems, A., & De Troch, M. (2014). Interactions between benthic copepods, bacteria and diatoms promote nitrogen retention in intertidal marine sediments. PloS one, **9(10)**, e111001.*

Conferences & Symposia

2018

British Phycological Society meeting 2018 (South-End, UK) - talk

Manton price (best oral presentation)

VLIZ Marine Science Day 2018 (Bruges, Belgium) 21 March 2018 - talk

Best oral presentation

TEDxGhent PhD Contest (Ghent, Belgium) 26 October 2018 - talk

2017

VLIZ Marine Scientist Day (Bruges, Belgium) 3 March 2017 - poster

2nd international MRM conference (Ghent, Belgium)7-8 September 2017 - talk

2016

VLIZ Marine Young Scientist's Day 2016 (Bruges, Belgium) 12 February 2016 - poster

second best scientific poster

Into the genome: advances in the world of algal genomics (Buckinghamshire, UK) 8-9 June 2016

1st International Conference on the Marine Microbiome, Discovery & Innovation (Berlin, Germany) 27-30 June 2016

Joint symposium on Eco-Evolutionary dynamics and Flanders Annual Meeting of Ecology (Ghent, Belgium) 20-21 December 2016 - talk

2015

VLIZ Marine Young Scientist's Day 2015 (Bruges, Belgium) 20 February 2015 - poster

second best scientific poster

5th International Student Conference on Microbial Communication 2015 (Jena, Germany) 13-16 April 2015 - talk

best oral presentation

Second EMBO Conference on Aquatic Microbial Ecology: SAME14 (Uppsala, Sweden) 23 August-28 August 2015 - talk

2014

Netherlands Annual Ecology Meeting (Lunteren, the Netherlands) 11-12 February 2014 - poster

VLIZ Marine Young Scientist's Day (Bruges, Belgium) 7 March 2014 - poster

PhD symposium, Faculty of Sciences, UGent, (Ghent; Belgium) 20 March 2014 - poster

Courses & Summer schools

2018

Holobiont workshop (EuroMarine network, Roscoff) France, 27-28 March 2018

Applying for a postdoctoral job *(*Doctoral Schools, Ghent)

2017

Tools for Multivariate Data Analysis (FLAMES,Ghent) 12-14 June 2017

2016

Continuous Data Analysis (MaStat, Ghent)

Black Forest Summer School: NGS data for phylogenetics (Phycomorph, Herzogenhorn , Germany) 13-16 September 2016

Microbial Communities: Modelling Meets Experiments (EMBL, Heidelberg, Germany) 7-11 December 2016

2014

Stable isotopes: analysis and application in food web ecology *(*Doctoral Schools, Ghent) 24-27 March 2014

Summer School 'Let's talk Science!' (VIB-Doctoral Schools, Brussels) 2-4 July 2014

Summer School Methodology & Statistics (FLAMES, Ghent)

Molecular and Physiological regulation of medical and environmental microbial biofilms (KULeuven, Vaalbeek) 15-18 September

An introduction to bioinformatics tools for metagenetic and population genomic data analysis (University of Gothenburg, Gothenburg, Sweden), 3-7 November 2014

2012

Summer school: Mathematical modelling (University of Nottingham, Nottingham, UK)

Grants & Funding

2018

BINCO expedition funding	NGO for biodiversity inventories

2017

FEMS funding for travelling	Overarching microbiology society

2016

FWO 'Hernieuwing' Aspirant	Flemish Government
Phycomorph funding for travelling	EU COST Action

2014

FWO Doctoral fellowship	Flemish Government

2013

BOF Doctoral Scholarship	Ghent University
Opwall funding for travelling	conservation & reserach organization
Francine Ronsse Award	Master dissertation award

2008

Laureate Frimout award — Final year project award

Teaching

Students supervised:

2014

Wannes Van Hoecke (3rd Ba Bio, bachelor dissertation)

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Frederik De Boever (2nd Ma Biology, master dissertation)

2015

Max Minne (3rd Ba Bio, bachelor dissertation)

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Coco Koedooder (2nd Ma in EMBC+, master dissertation)

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Courses taught:

Biodiversity Patterns in Space and Time (practicals)	(Ma Bio)
Biodiversity and Ecosystem Functioning (practicals)	(Ma Bio)
Algologie en Protistologie (practicals)	(Ba Bio)
Limnetische stage	(Ba Bio)
Algal Culturing Course	(doctoral school)

List of Abbreviations

ASW	Artificial Sea Water
BCC	Bacterial Community Composition
BI	Bayesian interference
C(CA)	(Constrained) Correspondence Analyses
chl	Chlorophyll
DAM	Diatom Artificial Medium
DAPI	4′,6-diamidino-2-phenylindole
DGGE	Denaturing Gradient Gel Electrophoresis
EDTA	Ethylenediaminetetraacetic Acid
EPS	Extracellular Polymeric Substances
F/2	Guillard's F/2 solution
F_0	Minimal Fluorescence
H	Shannon diversity index
HTP	High-Throughput
ITS	Internal Transcribed Spacer (region in the DNA)
ML	Maximum Likelihood
MPD	phylogenetic Mean Pairwise Distance
NMDS	Non-Metric Multidimensional Scaling
NSW	Natural Sea Water
OTU	Operational Taxonomic Unit
PAM	Pulse Amplitude Modulated
PAM	Pulse-Amplitude Modulated (fluorometry)
PCA	Principal Component Analysis
PCNM	Principal Coordinates of Neighbour Matrices
PERMANOVA	Permutational Multivariate Analysis of Variance
*rbc*L	RuBisCO Large Subunit (gene)
RDA	Redundancy Analysis
rDNA	Ribosomal DNA
TOM	Total Organic Matter
VIF	Variance Inflation Factor