Universität Bremen

# Investigation of diatom-bacteria interactions with the model organism *Thalassiosira rotula*

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# Summary

Marine microalgae are key primary producers responsible for more than 45% of global net primary production, fixing billions of tons of inorganic carbon each year. Diatoms constitute one of the most diverse and ecologically important group of microalgae. While diatom productivity and health are likely to be strongly governed by the structure and function of the diatom microbiome, we have little understanding which factors contribute to the microbiome assembly. In order to investigate the microbiome establishment on diatoms, an *in vitro* model system for reproducible laboratory studies was developed with the marine diatom *Thalassiosira rotula*. Thus, this thesis describes the isolation of diatoms and bacteria from the environment and the development of an *in vitro* model system for reproducible laboratory studies followed by the investigation of the microbiome assembling on the diatom *T. rotula* using co-culture experiments.

In **Chapter 2** diatoms and bacteria were co-isolated from a spring bloom in the German Bight of the North Sea. The isolation resulted in four different diatom species and 200 morphological different bacteria in culture. The marine diatom *Thalassiosira rotula* was selected as the model organism for the *in vitro* studies with diatoms and bacteria. **Chapter 3** focused on the development of a co-culture to study mutualistic interactions between the diatom *T. rotula* and bacteria as well as the generation of an axenic (bacteria-free) culture of the diatom *T. rotula*. The experiments revealed that the diatom *T. rotula* is auxotroph for B-vitamins and that the bacterial community of *T. rotula* is able to maintain the growth of the vitamin-free diatom with the provision of vitamins. In **Chapter 4 and 5** the microbiome assembling was investigated by exposing the vitamin-free and axenic diatom *T. rotula* to several bacterial source communities obtained from different diatom species. The co-culture experiments revealed that each of the newly established microbiomes on the *T. rotula* acceptor supports the growth of the diatom under vitamin absence, indicating that all microbiomes comprise bacteria capable for B-vitamin synthesis. To investigate the factors that contribute to the microbiome assembling, the bacterial community compositions of the different inoculated bacterial source communities and newly assembled acceptor microbiomes were analysed. The

analysis revealed that the different inoculated bacterial source communities were highly different in their bacterial community composition and contained up to 4406 different operational taxonomic units (OTUs). On the contrary, the analysis of the newly established acceptor microbiomes revealed that all acceptor microbiomes were similar to each other in respect to their bacterial community composition and that they were more similar to the original *T. rotula* bacterial source community than to the donor cultures where the bacterial source communities were obtained from. The similarity of the acceptor microbiomes was most likely caused by 10 OTUs, which constituted for more than 80% of the total relative abundance of all acceptor microbiomes. Furthermore, these 10 OTUs were shown to be most responsible for the differences between acceptor microbiomes and bacterial source communities and were thus described as the core microbiome of the diatom *T. rotula*. Consequently, it was shown for the first time that the ecologically relevant diatom *T. rotula* establishes a robust and reproducible bacterial core microbiome of 10 OTUs if it is offered highly diverse and compositionally different bacterial source communities with up to 4406 OTUs. The results of the robust and reproducible microbiome composition on the diatom *T. rotula* suggest that host factors contribute more than the bacterial diversity in the environment to the shaping of the microbiome composition.

# Zusammenfassung

Marine Mikroalgen sind wichtige Primärproduzenten, die jährlich Milliarden Tonnen anorganischen Kohlenstoff binden und für mehr als 45% der weltweiten Nettoprimärproduktion verantwortlich sind. Kieselalgen stellen eine der vielfältigsten und ökologisch wichtigsten Gruppe von Mikroalgen dar. Während die Produktivität und Gesundheit der Kieselalgen wahrscheinlich stark von der Struktur und Funktion des Kieselalgenmikrobioms beeinflusst wird, haben wir wenig Verständnis davon welche Faktoren zur Bildung des Mikrobioms beitragen. Um die Etablierung von Mikrobiomen an Kieselalgen zu untersuchen, wurde ein *in vitro* Modellsystem für reproduzierbare Laboruntersuchungen an der marinen Kieselalge *Thalassiosira rotula* entwickelt. Diese Arbeit beschreibt die Isolierung von Kieselalgen und Bakterien aus der Umwelt und die Entwicklung eines *in vitro* Modellsystems für reproduzierbare Laboruntersuchungen, gefolgt von der Untersuchung der Mikrobiom-Zusammenstellung auf der Kieselalge *T. rotula* mittels Co-Kultur-Experimenten.

In **Kapitel 2** wurden gleichzeitig Kieselalgen und Bakterien aus einer Frühjahrsblüte in der Deutschen Bucht der Nordsee isoliert. Die Isolation führte zu vier verschiedenen Kieselalgenarten und 200 morphologisch unterschiedlichen Bakterien in Kultur. Die marine Kieselalge *Thalassiosira rotula* wurde als Modellorganismus für die *in vitro* Untersuchungen mit Kieselalgen und Bakterien ausgewählt. **Kapitel 3** konzentrierte sich auf die Entwicklung einer Co-Kultur zur Untersuchung mutualistischer Wechselwirkungen zwischen der Kieselalge *T. rotula* und Bakterien sowie die Erzeugung einer axenischen (bakterienfreien) Kultur der Kieselalge *T. rotula*. Die Experimente ergaben, dass die Kieselalge *T. rotula* auxotroph für B-Vitamine ist und dass die Bakteriengemeinschaft von *T. rotula* in der Lage ist, das Wachstum der vitaminfreien Kieselalge durch die Zufuhr von Vitaminen aufrechtzuerhalten. In den **Kapiteln 4 und 5** wurde die Zusammensetzung des Mikrobioms untersucht, indem die vitaminfreie und axenische Kieselalge *T. rotula* mehreren Bakterienquellgemeinschaften ausgesetzt wurde, die von verschiedenen Kieselalgenarten gewonnen wurden. Die Co-Kultur Experimente zeigten, dass jedes der neu etablierten Mikrobiome auf dem *T. rotula*

Akzeptor das Wachstum der Kieselalge unter Vitaminmangel unterstützt, was darauf hindeutet, dass alle Mikrobiome Bakterien umfassten, die zur B-Vitaminsynthese fähig sind. Um die Faktoren zu untersuchen, die zum Aufbau des Mikrobioms beitragen, wurden die bakteriellen Gemeinschaftszusammensetzungen der verschiedenen geimpften bakteriellen Quellgemeinschaften und neu zusammengesetzten Akzeptormikrobiome analysiert. Die Analyse ergab, dass die verschiedenen geimpften bakteriellen Quellengemeinschaften in ihrer bakteriellen Gemeinschaftszusammensetzung sehr unterschiedlich waren und bis zu 4406 verschiedene operative taxonomische Einheiten (OTUs) enthielten. Im Gegensatz dazu ergab die Analyse der neu etablierten Akzeptormikrobiome, dass alle Akzeptormikrobiome in ihrer bakteriellen Gemeinschaftszusammensetzung einander ähnlich waren und dass sie der ursprünglichen *T. rotula* Bakterienquellgemeinschaft ähnlicher waren als denen der Spenderkulturen, aus denen die Bakterienquellgemeinschaften gewonnen wurden. Die Ähnlichkeit der Akzeptormikrobiome wurde höchstwahrscheinlich durch 10 OTUs verursacht, die mehr als 80% der gesamten relativen Häufigkeit aller Akzeptormikrobiome ausmachten. Diese 10 OTUs trugen auch am stärksten zu den Unterschieden zwischen Akzeptormikrobiomen und bakteriellen Quellgemeinschaften bei und wurden deshalb als Kernmikrobiom der Kieselalge *T. rotula* beschrieben. So konnte erstmals gezeigt werden, dass die ökologisch relevante Kieselalge *T. rotula* ein robustes und reproduzierbares bakterielles Kernmikrobiom von 10 OTUs etabliert, wenn ihr sehr unterschiedlich zusammengesetzte Bakterienquellgemeinschaften mit bis zu 4406 OTUs angeboten werden. Die Ergebnisse der robusten und reproduzierbaren Mikrobiomzusammensetzung auf der Kieselalge *T. rotula* deuten darauf hin, dass Wirtsfaktoren mehr als die bakterielle Vielfalt in der Umgebung zur Gestaltung der Mikrobiomzusammensetzung beitragen.

# Abbreviations

| | |
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| ANOSIM | Analysis of Similarity |
| ANOVA | Analysis of Variance |
| ASW | Artificial seawater |
| AWI | Alfred-Wegener-Institute Helmholtz-Centre for Polar- and Marine Research |
| $B_1$ | Vitamin $B_1$ (Thiamine) |
| $B_7$ | Vitamin $B_7$ (Biotin) |
| $B_{12}$ | Vitamin $B_{12}$ (Cyanocobalamin) |
| BAH | Biological Institute Helgoland |
| BEH | Ethylene Bridged Hybrid |
| BLAST | Basic Local Alignment Search Tool |
| bps | Base pairs |
| CC | *Cylindrotheca closterium* |
| CE | Collision energy |
| CS | *Chaetoceros socialis* |
| CV | Cone voltage |
| CAS | Chrome Azurol S |
| $CO_2$ | Carbon dioxide |
| DB | *Ditylum brightwellii* |
| DAPI | 4',6-diamidino-2-phenylindole |
| DNA | Deoxyribonucleic acid |
| DOC | Dissolved organic carbon |
| EDTA | Ethylenediaminetetraacetic acid |
| ESI | Electrospray ionization |
| ESAW | Enrichment medium of artificial seawater |
| $F_0$ | Minimal fluorescence yield of dark-adapted sample with all photosystem II centers open |
| $F_m$ | Maximal fluorescence yield of dark-adapted sample with all photosystem II centers closed |
| $F_v$ | Variable fluorescence of dark-adapted sample, $F_m - F_0$ |

| | |
|---|---|
| $F_v/F_m$ | Potential quantum yield |
| $H_2O$ | Water |
| HCl | Hydrochloric acid |
| HPLC | High Performance Liquid Chromatography |
| IAA | Indole-3-acetic acid |
| LB | Lysogeny broth |
| LC | Liquid Chromatography |
| MARUM | Centre for Marine Environmental Sciences, University of Bremen |
| MB | Marine broth |
| MS | Mass spectrometry |
| MPG | Max Planck Society for the Advancement of Science |
| MRM | Multiple reaction monitoring |
| NGS | Next-Generation Sequencing |
| OD | Optical density |
| OTU | Operational taxonomic unit |
| PS II | Photosystem II which shows variable fluorescence yield |
| PAM | Pulsed-amplitude-modulation |
| PCR | Polymerase chain reaction |
| PSU | Practical salinity unit |
| PCoA | Principal coordinate analysis |
| $r^2$ | Coefficient of determination |
| RDP | Ribosomal Database Project |
| RT | Retention time |
| rRNA | Ribosomal ribonucleic acid |
| SD | Standard deviation |
| SPE | Solid phase extraction |
| SSU | Small subunit |
| TEP | Transparent exopolymer particles |
| TP | *Thalassiosira pseudonana* |
| TR | *Thalassiosira rotula* |
| TW | *Thalassiosira weissflogii* |
| TRIS | Tris(hydroxymethyl)aminomethane |

| | |
|---|---|
| UPLC | Ultra Performance Liquid Chromatography |

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# Chapter 1

## 1 General Introduction

Marine microalgae such as coccolithophores, dinoflagellates, and diatoms are key primary producers (Field et al., 1998) responsible for more than 45% of global net primary production, fixing billions of tons of inorganic carbon each year (Falkowski and Raven, 2007; Simon et al., 2009). They are highly ecologically relevant because of their basal position in the marine food web (Fenchel, 1988).

Microalgae and bacteria coexist in the ocean since billions of years and their co-occurrence in a common habitat has encouraged interactions (Azam and Malfatti, 2007; Amin et al., 2012; Seymour et al., 2017). The long history of their interaction is evidenced from the large number of genes found in microalgae which presumably have their origin in bacteria, promoting the adaptation of algae to the conditions in the ocean (Armbrust et al., 2004; Bowler et al., 2008; Mackiewicz et al., 2013; Foflonker et al., 2018).

Recent research has shown that microalgae-bacteria interactions are complex, involving the exchange of micronutrients, cofactors, macronutrients, proteins, and signalling molecules. Bacteria are essentially dependent on autotrophic microalgae for organic carbon for their growth (Field et al., 1998; Falkowski et al., 2008), while microalgae need bacteria capable of remineralizing organic matter to inorganic compounds supporting algal growth (Worden et al., 2015). The diverse functions of microalgae-bacteria interactions highlight their relationship, covering obligate mutualism, commensalism, competition, and antagonism (Figure 1.1) (Kazamia et al., 2016; Seymour et al., 2017).

# 1.1 Microalgae-bacteria interactions

## 1.1.1 Parasitic interactions and defense

Microalgae-bacteria interactions have been frequently studied in the context of antagonistic or competitive relationships for nutrients (Bratbak and Thingstad, 1985), algicidal effects of bacteria (Mayali and Azam, 2004), and antimicrobial activity of microalgae (Findlay and Patil, 1984). For example, the Flavobacterium *Croceibacter atlanticus* infects the diatom *Thalassiosira pseudonana* by attachment to its surface and inhibition of cell division (Figure 1.1) (van Tol et al., 2017). This infection leads to cell elongation and plastid accumulation. Another example of the infection to diatoms by a member of the Bacteroidetes is the production of extracellular proteases by the bacterium *Kordia algicidal*, which causes algal cell lysis (Paul and Pohnert, 2011). The diatom *Chaetoceros didymus* responds to this attack of the bacterium with the production of algal proteases to defend itself against the bacterium. Moreover, the bacterial infection of microalgae is influenced by environmental parameters, for example Mayers et al. (2016) revealed that a representative of the genus *Ruegeria* sp. displays temperature-enhanced virulence to the microalgae *Emiliania huxleyi*.

## 1.1.2 Mutualistic interactions

In the last two decades, strong evidence of prevalent mutualistic interactions has changed the view that antagonism and competition are the most common associations between microalgae and bacteria (Croft et al., 2006; Cruz-López et al., 2018). Underlining support comes from frequent observations that microalgal growth declines in the absence of bacteria (axenic) under limiting or non-limiting nutrient conditions (Croft et al., 2005; Bolch et al., 2011; Windler et al., 2014; Bolch et al., 2017). This strong correlation of bacterial presence and microalgal growth suggests that bacteria contribute crucial compounds for microalgal growth. A well-recognized bacterial contribution to many microalgae species is the mutualistic interaction between B-vitamin-producing bacteria and auxotrophic microalgae that require these vitamins for growth and performance (Cole, 1982; Karl, 2002; Tang et al., 2010; Wagner-Dobler et al., 2010;

Kazamia et al., 2012; Grant et al., 2014; Cruz-López et al., 2018). Support for the B-vitamin dependency of microalgae comes from field work that revealed a substantial connection between vitamin exhaustion in seawater and microalgae bloom termination (Ohwada et al., 1972; Ohwada and Taga, 1972). Most microalgal species do not produce several essential vitamins. Croft et al. (2005) found that among 326 investigated microalgal species, more than 50% required cobalamin (vitamin $B_{12}$), 22% required thiamine (vitamin $B_1$) and 5% required biotin (vitamin $B_7$). Vitamin $B_1$ plays a central role in the general carbon metabolism and is a cofactor for several enzymes involved in branched-chain amino acid and primary carbohydrate metabolism. Vitamin $B_7$ is a cofactor for numerous fundamental carboxylase enzymes, involving acetyl coenzyme A carboxylase, which is in turn involved in fatty acid synthesis (Croft et al., 2006). Vitamin $B_{12}$ is required for the synthesis of amino acids, monosaccharides, and the reduction and transport of single carbon fragments in many biochemical pathways. For example, the corrinoid cofactor vitamin $B_{12}$ is only synthesized *de novo* by certain bacteria and archaea (Roth et al., 1996; Martens et al., 2002). Therefore, prokaryotes seem to be the ultimate source to sustain microalgal growth, since the ambient picomolar vitamin $B_{12}$ concentration in the marine environment is insufficient to sustain maximum microalgal growth (Bertrand et al., 2007; Koch et al., 2011).

Experimental evidence for the interaction between vitamin $B_{12}$-producing bacteria and auxotrophic microalgae has been shown by inoculating a bacterial culture of the genus *Halomonas* with a commercially available algal extract, whereby the bacterium increased the production of vitamin $B_{12}$ (Croft et al., 2005). Another example of this widespread interaction is between the cosmopolitan diatom *T. pseudonana* and the bacterium *Ruegeria pomeroyi*, a member of the genus *Roseobacter* (Durham et al., 2015). *R. pomeroyi* provides vitamin $B_{12}$ to *T. pseudonana* in exchange for a suite of microalgae-derived molecules, such as nitrogen compounds, sugar derivatives, and the 2,3-dihydroxypropane-1-sulfonate (DHPS) (Figure 1.1). A recent study revealed that the microalga *Ostreococcus tauri* interacts with the bacterium *Dinoroseobacter shibae*, a member of the *Rhodobacteraceae*, to obtain vitamins $B_1$ and $B_{12}$ (Cooper et al., 2018). In return, the microalga provides the bacterium with other B-vitamins, namely niacin ($B_3$), $B_7$, and p-aminobenzoic acid, which acts as a precursor for folate ($B_9$). The number of

microalgae-bacteria interactions focusing, among others, on the exchange of vitamins illustrates the importance of these micronutrients in the marine environment for auxotroph microalgae as well as bacteria.

Analogous to the well-known interaction between nitrogen-fixing bacteria and legumes in the terrestrial environment (Richardson et al., 2000) is the mutualistic interaction in the marine environment between nitrogen-fixing cyanobacteria and microalgae. The microalgae provide amino acids and a carbon source in return for nitrogen (Foster et al., 2011; Thompson et al., 2012; Hilton et al., 2013). A further example of a mutualistic interaction is the bacterial production of the plant hormone indole-3 acetic acid (IAA), which is a well-known plant growth promotor in the terrestrial ecosystem (Won et al., 2011; Fu et al., 2015). Amin et al. (2015) found that the diatom *Pseudo-nitzschia multiseries* secretes the amino acid tryptophan, which is converted by the bacterium of the *Sulfitobacter* species into IAA, which in turn is transferred back to the alga to stimulate its cell division and hence increase carbon production. The importance of this mutualistic interaction was underlined by the ubiquitous production of IAA in the ocean by members of the bacterial family *Rhodobacteraceae* (Amin et al., 2015; Simon et al., 2017) and by widespread growth responses of microalgae to IAA (Lau et al., 2009; Labeeuw et al., 2016; Segev et al., 2016).

Iron is an essential micronutrient for respiration and photosynthesis, and it limits bacterial growth and primary production because the bioavailable concentrations are insufficient to support algal growth in the ocean, thus making it a limiting resource (Coale et al., 1996). To increase the bioavailability of this key trace metal, many heterotrophic bacteria and cyanobacteria secrete siderophores, small organic molecules with exceptional high affinity for iron(III) (Vraspir and Butler, 2009). Bacteria transport the siderophores across the bacterial outer-membrane with carriers that are specific for various groups of siderophores (Moeck and Coulton, 1998; Hopkinson and Barbeau, 2012). In contrast, microalgae are not known to produce siderophores or to directly take up iron(III)-siderophore complexes of bacterial origin. However, genomic evidence suggests that numerous microalgae such as diatoms are capable to access iron from siderophores via ferrireductases and other iron(II) transporters on their outer cell

membranes (Kustka et al., 2007). Therefore, the bioavailability of iron for many microalgae species possibly depends on their association with bacteria. For example, bacterial siderophores are used by the microalga *Scrippsiella trochoidea* when produced in its environment (Amin et al., 2009). Bacteria from the *Marinobacter* species, which are often known to be associated with microalgae, produce the iron(III) complexing siderophore vibrioferrin. Vibrioferrin increases the availability of iron for *Marinobacter* during the night, but during the day when exposed to light the complex of vibrioferrin and iron degrades rapidly, releasing inorganic soluble iron to the water column. This unstable form of iron is then available for both bacteria and microalgae, and, in return, microalgae release dissolved organic carbon to support bacterial growth (Amin et al., 2009).

However, not only microalgae take an advantage from the association with bacteria, also bacteria benefit from dissolved organic carbon (DOC) compounds released by microalgae. Microalgae release excess carbon in form of polysaccharides, which constitute among other for a large fraction of microalgae-derived DOC (Underwood et al., 2010; Decho and Gutierrez, 2017). Diatoms, for example, secrete about 5% of their primary production as DOC, although photosynthesis spends a high demand of their energy (Wetz and Wheeler, 2007). Half of the DOC released by microalgae is consumed by bacteria (Azam et al., 1983) and consequently influences the community of heterotrophic bacteria which use these microalgae exudates (Grossart et al., 2005). The monomer composition of the microalgae exudates depends on the microalgae growth phase and species (Urbani et al., 2005). Correspondingly, it was shown that a nutrient shortage and thus a shift from exponential to the stationary growth phase stimulates the extracellular release of exudates (Grossart, 1999). Thus, nutrient limitation increases the release of polysaccharides in microalgae (Wetz and Wheeler, 2007). The increased exudation might serve to attract bacteria that are able to produce the limiting nutrient such as ammonium (Amin et al., 2015), vitamin (Croft et al., 2005; Croft et al., 2006) and iron (Amin et al., 2009). But not only microalgae actively control the exudation, also bacteria seem to impact on the quantity and the quality of microalgae exudates (Bruckner et al., 2011; Gärdes et al., 2012). Gärdes et al. (2012) showed, for example, that the Gammaproteobacterium *Marinobacter adhaerens* in co-culture stimulates the production

of transparent exopolymer particles (TEP) of the diatom *Thalassiosira weissflogii*, which in return can be used by the bacterium as a carbon source.

### 1.1.3 Other interactions

In addition to distinct parasitic and mutualistic interactions, the interactions between microalgae and bacteria have been shown to vary during different growth phases of algae. For instance, *Phaeobacter gallaeciensis*, a member of the class Alphaproteobacteria, forms a possibly mutualistic association with healthy cells of the coccolithophore *E. huxleyi* by supporting the microalgae with the hormone phenylacetic acid and the antibiotic tropodithietic acid, potentially against antagonistic bacteria, in return for a carbon source (Seyedsayamdost et al., 2011). Upon the release of p-coumaric acid by the microalga, a product of senescence, the bacterium changes into an antagonistic lifestyle and releases algicidal molecules that lyse *E. huxleyi*. A similar interaction has been described between *D. shibae* and the microalgae *Prorocentrum minimum* (Wang et al., 2015). This "Jekyll and Hyde" phenomenon allows the bacterium to maximize the output of microalgal organic matter for its benefit, independent of the physiological state of the host. The prevalent observations of the "Jekyll and Hyde" strategies with Alphaproteobacteria suggest that this type of interaction is widespread in this bacterial class.

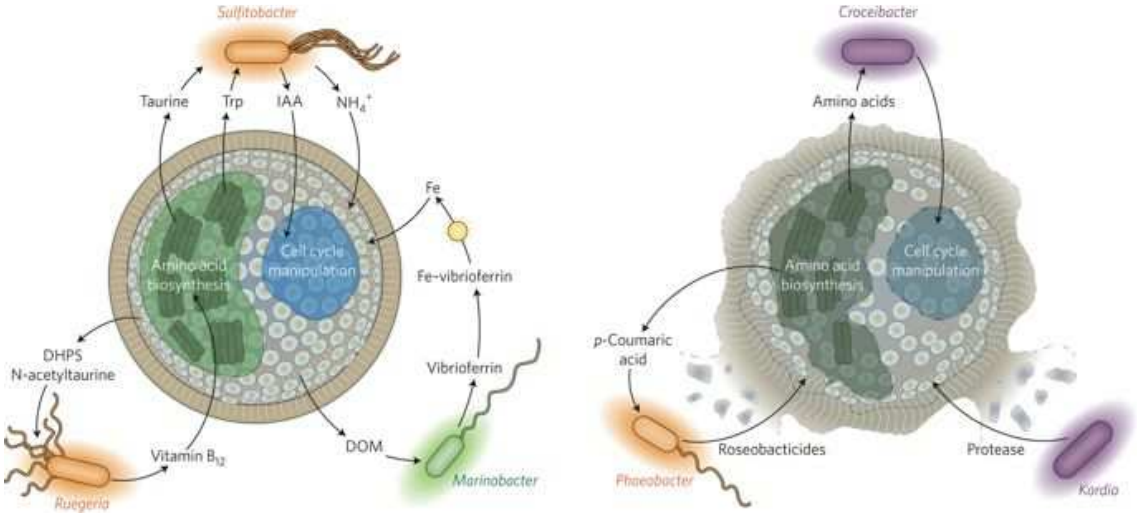



Figure 1.1. Mutualistic (left) and algicidal (right) microalgae-bacteria interactions in the algal phycosphere (adopted from Seymour et al. (2017)). Bacteria are colour-coded according to their phylogenetic classification: *Rhodobacteraceae* in orange, *Alteromonadaceae* in green and *Flavobacteriaceae* in purple. A general microalgal cell is shown and symbolize multiple species. A shadow around microalgae or bacteria represents the gradient of molecules diffusing out of the algal cells. Mutualistic interactions (left) occur between microalgae and *Sulfitobacter*, *Ruegeria* and *Marinobacter*. *Sulfitobacter* increases the growth of the diatom *P. multiseries* by converting diatom-derived tryptophan (Trp) to the growth-promoting hormone indole-3-acetic acid (IAA), which is released and subsequently taken up by the diatom to increase its cell division. *Sulfitobacter* also provides ammonium ($NH_4^+$) to *P. multiseries* in exchange for the diatom-secreted carbon source taurine. *R. pomeroyi* provides the diatom *T. pseudonana* with vitamin $B_{12}$, which is used in biosynthesis of the amino acid methionine in exchange for several carbon sources, including N-acetyltaurine and 2,3-dihydroxypropane-1-sulfonate (DHPS). *Marinobacter* secretes the siderophore vibrioferrin to acquire iron in the dark; in sunlight, the iron–vibrioferrin complex is highly photolabile and degrades, releasing bioavailable iron that is taken up by microalgae in exchange for DOM. Algicidal interactions (right) take place between microalgae and *Croceibacter*, *Phaeobacter* and *Kordia*. *C. atlanticus* attaches to diatom cell surfaces and releases an unidentified molecule that blocks diatom cell division and increases diatom secretion of organic carbon, including amino acids. *P. gallaeciensis* senses secretion of p-coumaric acid from the coccolithophore *E. huxleyi* during senescence, which activates the bacterial production and release of the algicidal molecules roseobacticides A and B, which lyse *E. huxley*i and release DOM. *K. algicida* produces extracellular proteases that lyse diatom cells in order to acquire DOM.

### 1.1.4 The phycosphere

Microalgae-bacteria interactions are suggested to occur in a region surrounding individual microalgal cells. This microenvironment, termed the phycosphere, extends outwards from the microalgal cell to some distance where algal exudates are still available (Bell and Mitchell, 1972; Seymour et al., 2017). The phycosphere is therefore the marine counterpart to the rhizosphere in soil ecosystems and has direct influence on nutrient exchange of microalgal cells (Figure 1.2). The phycosphere occurs because all aquatic cells are enclosed by a coat of fluid known as the boundary layer. In the case of a microalgal cell, the boundary layer is not mixed with the bulk water because turbulence does not happen at a scale less than ~100 µm (Lazier and Mann, 1989). Microalgae enrich the phycosphere by the active exudation of photosynthetically fixed carbon compounds. These exudates consist to a large extent of high molecular weight (HMW) carbon compounds (Hansell, 2013) like polysaccharides and to a lesser extent to other carbon compounds with differing molecular weights. The reason for that is the diffusion rate of compounds in the phycosphere largely depends on the molecule size and HMW compounds have therefore a longer duration of stay (Seymour et al., 2017). The microalgae-derived polysaccharides are fundamental in the attraction and maintenance of bacteria in the phycosphere (Bell and Mitchell, 1972; Seymour et al., 2010; Sonnenschein et al., 2012). Inside the phycosphere, bacteria are recruited by the high concentration of alimentary compounds (Stocker, 2012) and may perform several advantageous roles for microalgae, such as the remineralization of the released carbon compounds to make them available again for primary production (Amin et al., 2012; Seymour et al., 2017).

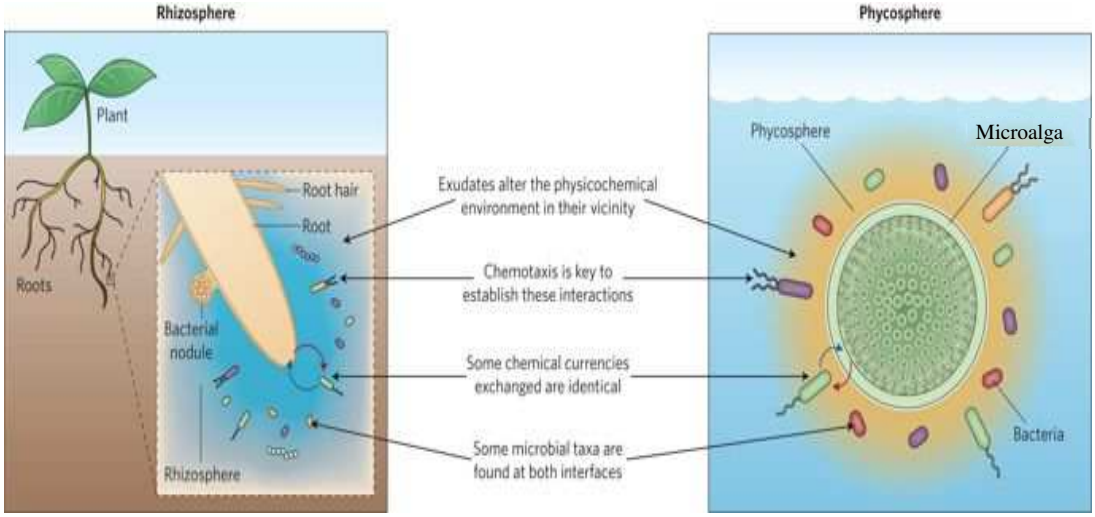



Figure 1.2. The rhizosphere and the phycosphere are analogous microenvironments. The phycosphere, defined as the region immediately surrounding a microalgal cell that is enriched in organic molecules exuded by the cell, is a key microenvironment for planktonic aquatic bacteria. It is the aquatic equivalent of the rhizosphere, which is an essential region for plant-microorganism interactions in terrestrial habitats. This figure was adopted from Seymour et al. (2017).

The availability of microalgal products inside the phycosphere is not only steered by mass diffusivity of the compounds, but also by the capability of bacteria to detect and recruit this zone. Bacteria reach the phycosphere either by chemotaxis, motility, vertical transmission, or by random encounter (Seymour et al., 2017). The differences in availability of extracellular products by bacteria can be underlined by two different proposed modes of exchange of nutrients between microalgae and bacteria (Karl, 2002). One suggested method is that bacteria may attach to the phycosphere and exchange nutrients directly with microalgae without losing nutrients to the surrounding water. The other method proposed for the exchange is indirect: microalgae excrete the nutrients into the surrounding water and free-living bacteria access the nutrients indirectly from the water column. Both direct (Croft et al., 2005) and indirect (Kazamia et al., 2012) exchange of nutrients between microalgae and bacteria are described in the literature. However, it might be more advantageous for bacteria to colonize the phycosphere immediately, because a non-motile bacterium will only encounter ~0.0035 microalgae cells per day, whereas a motile bacterium will hit on ~9 microalgae cells per day (Seymour et al., 2017). Therefore, the preferred method of exchange between microalgae

and bacteria possibly depends on the ability and opportunity of bacteria to recruit the phycosphere.

The function of microalgae in shaping the bacterial recruitment of their phycosphere is not yet fully understood just like the way bacteria influence their colonization into the phycosphere. Until now, it has only been demonstrated that microalgal-associated bacteria are mostly limited to specific phyla of the Proteobacteria (*Sulfitobacter*, *Roseobacter,* and *Alteromonas*) and, to a lesser extent, Bacteroidetes (*Flavobacterium*) (Schafer et al., 2002; Croft et al., 2005; Grossart et al., 2005; Sapp et al., 2007; Amin et al., 2015). These studies provided initial indications that microalgae and / or bacteria might influence the recruitment since the different examined microalgae species harboured similar bacterial taxa in their communities. Hence, more research is required to investigate if microalgae harbour a specific bacterial community shaped by the algal host and / or the bacteria.

## 1.2 Microalgae microbiome

So far, most investigated interactions between algae and bacteria have been studied with *in vitro* co-culture experiments using single bacterial species and microalgae. Modern next generation sequencing (NGS) tools like amplicon-based sequencing, metagenomic, and metatranscriptomic studies instead focus on the entire bacterial community associated with microalgae. The analysis of bacterial communities has the advantage that it is not limited by the cultivation of bacteria. Research with focus on the microalgae-associated bacterial communities can be divided into mechanistic studies, investigating their function, and descriptive studies, investigating who is present in a community. Cruz-Lopez and Maske (2016) sequenced a newly established associated bacterial community on an axenic and vitamin-depleted *Lingulodinium polyedrum* culture. The inoculated bacterial community was obtained from natural seawater and supported the growth of the dinoflagellate by producing vitamin $B_1$ and $B_{12}$. Similar to many other studies performed before with single bacterial isolates (reviewed in Croft et al., 2006), they revealed that microalgae receive B-vitamins from their associated bacteria. However, they did not investigate which bacteria in the community synthesized

the vitamins. Krohn-Molt et al. (2017) provided strong evidence by metagenomics and transcriptomics that the B-vitamin supply is carried out by a rather small group of bacteria in the microalgae-associated bacterial community. Furthermore, they showed that different bacterial species produced the B-vitamins in the three investigated freshwater microalgal species, including *Chlorella saccharophila*, *Scenedesmus quadricauda* and *Micrasterias crux-melitensis* (Krohn-Molt et al., 2017). Recent research increasingly concentrates on descriptive investigations of microalgae-associated bacterial communities to better understand the bacterial composition in the phycosphere. Thus, these studies reveal that different microalgae harbour a unique and specific bacterial community (Grossart et al., 2005; Krohn-Molt et al., 2017; Behringer et al., 2018; Crenn et al., 2018).

Cultivation of microalgae is essential to study microalgae-bacteria interactions. To perform such experiments, cultures are often obtained from culture collections or kept in cultivation in the laboratory for months or years. However, culturing under nutrient-rich laboratory conditions is problematic as it provides essential nutrients in exceeding natural conditions thus potentially leading to changes in the microalgae microbiome. Behringer et al. (2018) demonstrated the opposite, supporting many investigations performed with long-term cultivated microalgal species and their associated bacterial communities. They showed that the bacterial communities associated with various strains from the diatoms *Asterionellopsis glacialis* and *Nitzschia longissima* showed high conservation across strains at the genus level, and that long-term cultivation (>1 year) resulted only in small changes in the microbiomes. Moreover, a recent study revealed that the sampling location and sampling season of microalgae have a more significant influence on the microalgae-associated bacterial community than phylogenetic affiliation (Ajani et al., 2018). However, except for a few studies (e.g. Ajani et al., 2018), it appears that microalgae harbour a unique and distinct microbiome. Despite the identified specificity of microalgae microbiomes, information on how they get shaped are still scarce. Until now, it is only described in the literature how abiotic factor shape the microbiome, but little is known about how biotic factors like microalgae and bacteria influence the composition and specificity of microalgae microbiomes. The elucidation

how these interactions are mediated in the phycosphere is of global significance, because their interactions drive the carbon cycling and the productivity of the ocean.

## 1.3 Aims and scientific objectives

This thesis aimed to study the associations of diatoms and bacteria in relation to their mutualistic interactions. The experimental design involved the isolation of microalgae and bacteria samples from the environment with the goal to develop an *in vitro* model system for reproducible laboratory studies to study the core microbiome on selected microalgae. The work for this thesis was carried out in two different laboratories. The main part was done in the working group 'Marine Chemistry' of Prof. Dr. Tilmann Harder at the University of Bremen. The other part was done in the lab of Associate Professor Dr. Rebecca Case at the University of Alberta during a 3-month research stay in Canada. Specific methods were trained in the lab of Dr. Case like the pulsed-amplitude-modulation fluorometry and imaging flow cytometry to determine microalgae and bacteria abundance and concurrently algal physiology.

The specific objectives of this thesis were as follows:

The first objective was to co-isolate bacteria and microalgae and to establish permanent cultures in the laboratory. The co-isolation of microalgae and bacteria of the same spatial and temporal context is important because the place of origin and time of isolation of microalgae play an important role in shaping the microbiome. To be able to compare the microbiome of different microalgal species, the samples of microalgae have to be isolated altogether from the same location and time.

The second objective was to develop a co-culture system to study mutualistic associations between microalgae and bacteria. A co-culture set-up is essential to run experiments under reproducible conditions while individual parameters (e.g., B-vitamins) can be manipulated. Such targeted manipulations allow to study the interactions of both organisms based on particular micronutrients. The co-culture system can be used for a range of different experiments, such as investigating the mutual exchange of siderophores

or B-vitamins in return for DOC. The following hypotheses were addressed within the second objective: (A) It is possible to establish axenic cultures of the diatoms *Thalassiosira rotula*, *Ditylum brightwellii*, *Cylindrotheca closterium* and *Chaetoceros socialis*. (B) The diatom *T. rotula* is auxotroph for B-vitamins. (C) The bacterial community of *T. rotula* supports the growth of vitamin depleted *T. rotula* cells by the provision of B-vitamins.

The final objective was to describe the core bacterial community associated with the diatom *T. rotula* and the role of microalgae and bacteria in shaping the specific microbiome. To investigate the formation of a core microbiome, axenic microalgae are inoculated with different diatom bacterial source communities and the phylogeny of the donor and acceptor cultures of the inoculation are analysed. Together with the final objective the following hypotheses were addressed: (A) The associated bacterial communities of the acceptor cultures share certain bacterial taxa. (B) The diatom *T. rotula* harbours a core microbiome.

# Chapter 2

# 2 Isolation and culturing techniques for microalgae and bacteria

## 2.1 Introduction

The objective of this chapter was to isolate microalgae and bacteria from the same environment to develop an *in vitro* model system for reproducible laboratory studies to explore the core microbiome of selected microalgae. The sampling site was the island of Helgoland in the German Bight. Water samples were collected at 5 - 7 consecutive timepoints between March and May during the spring bloom 2016, depending on whether microalgae or bacteria were isolated.

This chapter describes the method development to run co-culture experiments of microalgae with bacteria.

## 2.2 Methods

### 2.2.1 Sample collection

5 L seawater was collected at seven timepoints between March and May 2016 at the island of Helgoland in the German Bight (54° 11'03" N, 7°54'00" E) (Figure 2.1). Sampling was carried out with the research vessels Diker or Aade of the "Biological Institute Helgoland (BAH) / Alfred Wegener Institute, Helmholtz-Centre for Polar- and Marine Research (AWI)". The samples were taken in upwind direction with an acid rinsed Niskin-Type Plastic Water sampler PWL (5 L model) at 1 m depth while the boat was drifting in the current. Samples were taken according to GEOTRACES protocols (http://www.geotraces.org/images/Cookbook.pdf, last time opened 14.07.2019, 12:01 pm). Environmental conditions at the sampling site ranged from 5.9 to 10.6°C and 32.1 to 33.6 PSU during the 3-month sampling period (Figure 2.2).


Figure 2.1. Map of the German Bight. The inlay shows a complete map of Helgoland and the asterisk indicates the sampling site in spring 2016. Google Maps©

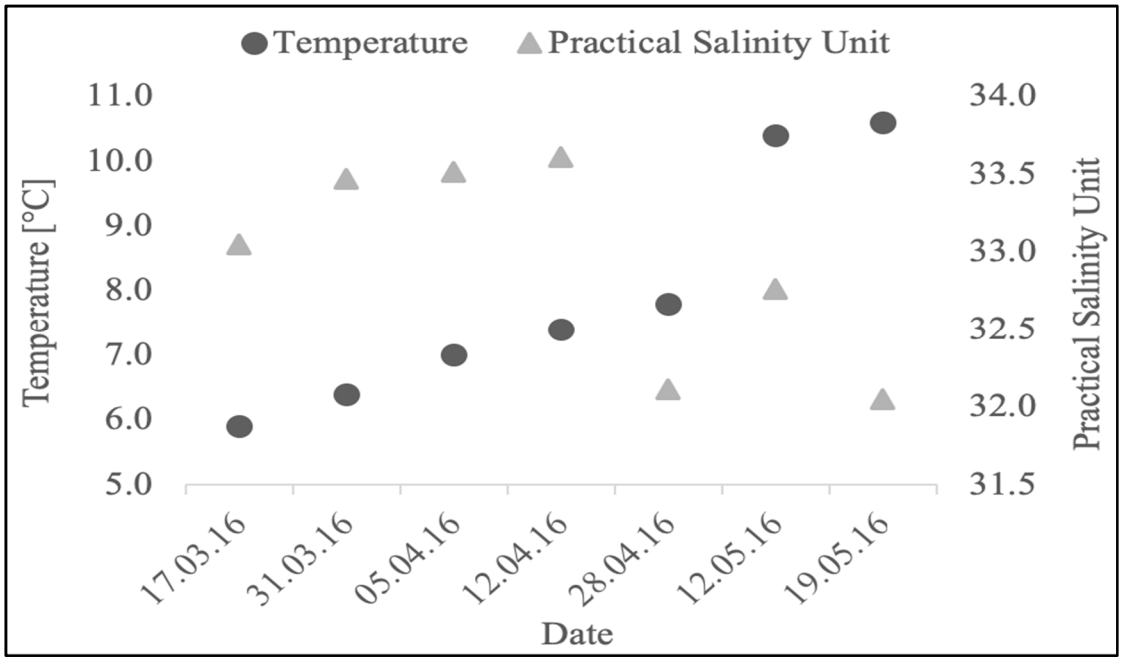



Figure 2.2. Temperature and salinity progression during the seawater sampling from March till May 2016 at the island of Helgoland in the German Bight.

### 2.2.2 Isolation, identification and cultivation of microalgae

An aliquot of 50 mL seawater was taken from the 5 L samples on the dates 17.03.2016, 31.03.2016, 12.05.2016 and 19.05.2016 (Figure 2.2). To isolate microalgae, 1 mL seawater was diluted in 4 mL sterile enrichment medium of artificial water (ESAW) (Harrison et al., 1980) (Supplementary information) in 12-well plates. Single cells or chains were picked under the light microscope (Primovert, Zeiss) with a micropipette and transferred to a new well containing sterile ESAW medium. The cells were grown for 2 d at 15°C (MIR 254 PE, Panasonic) under a 12 h light/12 h dark diurnal cycle (30 - 70 µmol photons $m^{-2}$ $s^{-1}$) (Sun Strip "daylight", Solar Stinger LED) and checked under the microscope for purity. The purity of the microalgal cultures was assessed by screening 1 mL per culture 3 times for contamination with morphologically different cells. The screening was conducted 3 d after single cell isolation and again after 2 and 3 weeks. In case of impurity, the steps were repeated until a unicellular culture was obtained. Microalgae were examined by light microscopy (Primovert, Zeiss) and referenced to phytoplankton identification literature from the North Sea (Kraberg et al., 2010).

On 03.09.2017 another microalga was isolated from a 100 µm plankton net haul collected around Helgoland. The isolation was performed as described above, however this time the alga was grown in both ESAW replete and deplete of the vitamin stock. Post isolation, these cultures were maintained under the respective conditions.

The culture conditions for microalgae were adopted from the literature (Schone, 1972). The broad-spectrum artificial seawater medium ESAW was selected to culture the microalgae in a reproducible manner and under defined nutrient conditions.

The culture conditions such as temperature, light and the culture medium described in the section 2.2.2 are referred to as regular growth conditions in the following or indicated if different.

### 2.2.3 Isolation and classification of bacteria

Polycarbonate filters (Whatman™, Nucleopore, 46 mm) with pore sizes of 0.2, 5 and 10 µm were placed for 72 h in 6 N HCl and subsequently stored in $ddH_2O$ at pH 2 (slightly acidified with HCl) until use. The filtration units were rinsed with 200 mL $ddH_2O$ and 200 mL HCl 0.01 N. The filtration units with inlayed filters were rinsed again with 100 mL $ddH_2O$. Approximately 500 - 1000 mL seawater was filtered through the filters depending on particle concentration of the samples. Clogged filters were replaced until the final volume was processed. Each filter was transferred to sterile Eppendorf tubes containing 1 mL of 0.2 µm pre-filtered artificial seawater (35 g/L Instant Ocean® Sea salt) and vortexed to suspend bacteria and phytoplankton. The suspensions were first diluted serially up to $10^{-4}$ and 100 µL of each dilution was spread plated on solid growth media (Table 2.1). The following solid media were used: 1/10 marine broth (Supplementary information), minimal iron (Supplementary information) and minimal-iron-EDTA (Supplementary information). CAS medium (Supplementary information) was used to isolate bacteria that are capable to produce siderophores. CAS agar is a marine agar that additionally contains a stain that can be used for detection and determination of siderophores (Schwyn and Neilands, 1987).

Table 2.1. Solid growth media used to isolate bacteria from seawater. The plus (+) indicates which medium was used on the specific date.

| Date | Solid growth medium | | | |
|---|---|---|---|---|
| | 1/10 marine broth | Minimal iron | Minimal-iron-EDTA | CAS |
| 17.03.2016 | + | + | | |
| 05.04.2016 | + | + | | |
| 12.04.2016 | + | + | + | |
| 28.04.2016 | + | + | + | |
| 12.05.2016 | + | + | + | |
| 19.05.2016 | + | + | | |
| 03.07.2016 | | | | + |

Single bacterial colonies were picked with sterile pipette tips under the binocular microscope (Stemi, Zeiss) and quadrant streaked on the different solid growth media until a pure culture was obtained. A culture was considered pure if a colony was twice quadrant streaked and if both times all colonies appeared the same (e.g. colour and colony shape). A single colony was grown overnight in the liquid medium corresponding to the isolation medium and bacterial glycerol stocks were created from the overnight culture with a mixture of 500 µL bacteria culture and 500 µL sterile 50% glycerol. All stocks were stored at -80°C until further experiments. Bacteria obtained from glycerol stocks were typically grown either on marine agar plates incubated at room temperature in the dark or in marine broth for 12 - 36 h (room temperature, 150 rpm), unless stated otherwise.

To isolate genomic bacterial DNA, single bacterial colonies were taken from agar plates and transferred to 50 µL TRIS-EDTA buffer. Samples were boiled in the buffer for 4 min by 95°C and subsequently cooled down to 4°C until further use. After centrifugation for 1 min at 3000x g, the supernatant was collected and the DNA extract was stored at -20°C. Amplification of 16S rRNA genes was performed using the universal bacterial primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Weisburg et al., 1991). The PCR reactions (25 µL) contained 2 - 10 ng of the extracted genomic DNA, 200 µM of each primer, 0.2 mM of each dNTP, 2.5 µL 10x DreamTaq reaction buffer and 0.75 µL DreamTaq DNA polymerase (Thermo Fisher Scientific EP0701, 5U/µL). The PCR was performed (Perkin Elmer, GeneAmp PCR System 2400) with an initial denaturing cycle at 95°C for 3 min, followed by 30 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 1 min and a final extension cycle at 72°C for 10 min. The DNA quality was verified by agarose gel electrophoresis (Life technologies Inc., Horizon® 58). The 1.5% agarose gel was stained with DNA Stain G (SERVA) and DNA was stained with DNA gel loading dye (6X) (Thermo Fisher Scientific). The size of the DNA fragments in the samples were compared to GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific). Amplified products were cleaned using the Wizard gel and PCR clean up system (Promega). Purified PCR products were sequenced with the 27F primer using Sanger technology (GATC Biotech).

Sequences were quality-trimmed using Sequencher 5.6 (Gene Codes) and pairwise aligned using CLUSTALW (www.genome.jb/tools-bin/clustalw). Two unrooted pairwise aligned phylogenetic trees were created in R (R Core Team, 2018) using the packages APE (Paradis et al., 2004) and GEIGER (Harmon et al., 2008).

### 2.2.4 Measuring microalgal and bacterial growth

To perform co-culturing experiments with microalgae and bacteria, methods for the determination of the growth of microalgae and bacteria were adopted or modified from published protocols.

A Sedgwick-rafter counting chamber (spi Supplies) was used to count microalgae. Microalgae were counted under 100x magnification with an inverted microscope (Primovert, Zeiss). Samples were fixed and stained with Lugols iodine solution (Supplementary information) and a minimum of 400 cells/mL were counted. In case of fewer than 400 cells/mL, the complete area of the counting chamber (50 mm long x 20 mm wide and 1 mm deep, volume 1 mL) was enumerated.

However, this technique was unsuitable to count many replicated experiments ($n > 18$), since microscope-based cell counting is time consuming and many of the assays required daily measurements to estimate when to apply the next dilution steps or to replenish micronutrients. Therefore, different strategies were tested to correlate algal cell densities with other experimental proxies of cell abundance.

#### 2.2.4.1 Determining the chlorophyll fluorescence of microalgae by fluorescence

To measure the total chlorophyll fluorescence in a small volume, microalgae cultures were measured in a fluorescence plate reader. Microalgal growth was monitored using a filter-based fluorescence plate reader (FLUOstar Omega, BMG). Relative fluorescence units (RFU) were measured in black 96-well polystyrene microplates with clear bottom using optical filters at 440-80 nm for excitation and 640-80 nm for emission.

A volume of 150 µL was used to measure the RFUs. Prior to the first fluorescence measurement, the gain was set to 10% and was used for all upcoming measurements. To ensure the same function of the plate reader over time, a chlorophyll standard was measured in parallel. RFUs of samples were measured in top and bottom mode and averaged across both values. The measurement was done in the well scan mode with 5x5 matrix and a scan mode of a 6 mm diameter. Using this method, it was possible to determine the RFUs in a 96-well plate in about 20 min.

To validate the fluorescence measurement with a plate reader, the microalgae abundance was measured in five 1:2 serial dilution steps of a *Thalassiosira rotula* culture with a plate reader and in parallel counted under the microscope. The linear relationship between RFUs and cell counts was determined by Pearson's correlation.

The determination of the microalgal biomass was done either by counting the cells under the microscope or by measuring the RFUs with a fluorescence-based plate reader. However, only the biomass change over time could be detected, whereas the physiology of algal cells was inaccessible by these methods. Therefore, a method to determine the microalgae cell density as well as the physiological state of the cells using fluorescence measurements was used.

Samples were taken 5 - 7 h into the light cycle and diluted in ESAW medium to be within the detection range and measured using a pulsed-amplitude-modulation (PAM) fluorometer (Water-PAM, Walz). A dark adaptation period of 20 min with the lowest stirring adjustment was used, after which a saturating pulse was applied, and fluorescence readings were taken to measure the minimal dark fluorescence ($F_0$) that is directly correlated to the chlorophyll content as well as the maximum dark fluorescence ($F_m$). The photosystem II (PSII) potential quantum yield is a normalized ratio and is created from the minimal and maximal dark fluorescence ($F_v/F_m = (F_m-F_0)/F_m$). $F_v/F_m$ is a measurement ratio that represents the maximum potential quantum efficiency of PSII if all capable reaction centres were open and reveals with it information about the physiological status of the cells (Schreiber et al., 1986; Vankooten and Snel, 1990).

To validate the fluorescence measurement with a PAM fluorometer, the abundance of the diatom *T. rotula* was measured in a growing culture every 24 - 48 h for 6 d with a PAM fluorometer and in parallel counted under the microscope. The linear relationship between $F_0$ and cell counts was determined by Pearson's correlation.

### 2.2.4.2 Determining bacterial cell densities

To quantify the cell density of bacteria, the optical density (OD) of a bacterial suspension was measured in a spectrophotometer (DU 640, Beckman) at 600 nm. Marine broth was used for the dilutions and as a blank. A disadvantage, however, is that the OD measurement cannot be compared between different strains of bacteria as each strain may cause a different light scattering. For co-culture experiments with microalgae and bacteria, it was required to adjust exact bacterial cell numbers/densities in microalgal cultures. Therefore, DAPI stained bacterial cells were counted under the fluorescence microscope.

1 mL of a bacterial suspension was vacuum filtered over a black 0.2 µm pore-size polycarbonate membrane filter (Whatman® Nucleopore Track-Etched Membranes). Subsequently, this filter was stained with DAPI (4', 6-diamino-2-phenylindole) (VECTASHIELD Antifade Mounting Medium) and bacteria were counted under the epifluorescence microscope (Axioskop 2, Zeiss) with filters ($\lambda_{ex}$ 350 nm, $\lambda_{em}$ 460 nm) and 630x or 1000x magnification using an eyepiece with an integrated counting grid (10 x 10, 1.25 $mm^2$). A minimum of 10 counting grids were enumerated in each sample and cell counts were extrapolated to obtain the number of cells per mL.

To validate the OD measurement of bacteria, the bacterial cell density was determined in parallel by photometry and epifluorescence microscopy of DNA-stained cells. The strain *Marinobacter* sp. (Mar1 74DC42, phylogenetic tree, Figure 2.5B) was grown in marine broth at the regular growth conditions for 24 h (Section 2.2.3) and determined in one 1:4 and five 1:10 serial dilution steps respectively. The linear relationship between OD and cell counts was determined by Pearson's correlation.

#### 2.2.4.3 Determining cell densities of bacteria and microalgae using image flow cytometry

The photometric and fluorometric methods correlated bacterial and microalgal cell numbers only with quantitative reference data. A direct assignment to cell numbers was not possible because each species might have a different light absorbance or a different amount of chlorophyll. Flow cytometry allows simultaneous cell counting of bacteria and microalgae in the same sample, thus saving time and sample volume.

Microalgae were fixed with 0.15% glutaraldehyde (Sigma-Aldrich) by incubating cells in the dark for 10 min, then flash-frozen in liquid nitrogen and stored at -80°C until flow cytometry was performed using an ImageStream®$^X$ Mark II Imaging Flow Cytometer (Amnis Corporation). Samples were stained with SYBR Green (Sigma-Aldrich) 15 min prior to measurement. A 488 nm laser was used for excitation. Another measurement was done simultaneously using chlorophyll fluorescence (642 nm) and FITC fluorescence (560 nm) for detection of microalgae and bacteria, respectively. Microalgae were gated based on present chlorophyll fluorescence and images in 20x magnification to distinguish the chain length of the microalgae. Bacteria were gated based on a high SYBR Green staining and low side scatter.

To validate microalgae cell counts in the imaging flow cytometer, *T. rotula* cultures were grown under regular growth conditions (Section 2.2.2) for 6 d in 6-well plates. Samples were enumerated in parallel via flow cytometry and microscopy. The linear relationship between cytometric and microscopic cells counts was determined by Pearson's correlation.

### 2.2.5 Monitoring the growth of algal cultures

To conduct culture experiments with microalgae, the growth characteristics in the different culture containers such as 20 mL culture flask, 96-well plates, 12-well plates as well as 1 L glass bottles were compared.

The ESAW medium was prepared at the normal and the 10x concentration of all ingredients except for sodium chloride, magnesium chloride, potassium chloride, calcium chloride and sodium sulphate. The rationale behind increasing the nutrients in the ESAW medium was to delay the exhaustion of nutrients in small volumetric 96-well plates. It is hypothesized that the increase of nutrients in the medium delays the nutrient shortage in 96-well plates and 20 mL culture flasks.

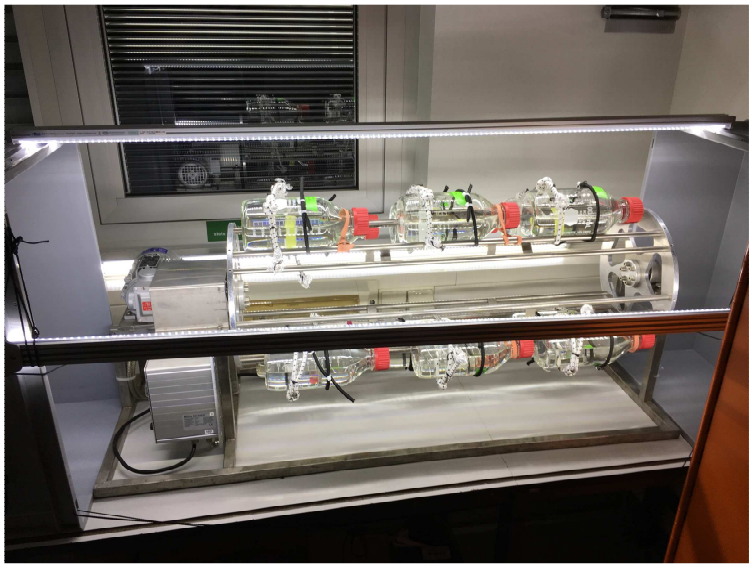
To compare diatom growth characteristics in 96-well plates (n = 4) and 20 mL culture flasks (n = 3), *Cylindrotheca closterium*, *Chaetoceros socialis*, *Ditylum brightwellii* and *T. rotula* cultures were inoculated from a mid-exponential phase into fresh medium (1x & 10x concentration) to an initial cell density of ~1500 - 2500 cells/mL in both culture containers. The cultures were grown under the above described growth conditions (Section 2.2.2). The outmost rows of the 96-well plate were left empty in order to avoid evaporation according to Sher et al. (2011). Samples were transferred from the flask to the plate for the measurement or directly measured in the plate.

To compare diatom growth characteristics in 12-well plates and 20 mL culture flasks, *T. rotula* cultures were inoculated from a mid-exponential phase culture in fresh medium to an initial diatom cell density of ~2000 cells/mL in both culture container (n = 3). The cultures were grown at the above described growth conditions (Section 2.2.2). The flask was re-sampled and the wells in the microplate were only once sampled for the fluorescence measurements.

The used microplate protocol is an established and published method to perform microalgae growth experiments without re-sampling of the same microplate well (Bramucci et al., 2015). Data points obtained by this method were connected by lines to a growth curve, although the data were not obtained by re-sampling.

To be able to culture algae in larger volumes, 1 L glass bottles were tested for cultivation. The cultivation was performed on a roller table to avoid shading of the microalgal cells at the bottom of the bottle and to simulate turbulence. *T. rotula* was grown in triplicate in a 1 L glass bottles (DURAN® laboratory bottle with DIN thread,

GL 45) and rotated with 6.2 rpm on a roller table (Figure 2.3). In the 1 L glass bottle method the microalgae were grown in a culture volume of 700 mL and for each cell counting 100 - 1000 µL culture were repeatedly removed from the same bottle. *T. rotula* cultures were inoculated from a mid-exponential phase growing culture into fresh medium to an initial diatom cell density of ~2000 cells/mL. The culture volume was kept at 700 mL to enable water movement in the bottle. The cultures were grown under the regular light conditions (Section 2.2.2) with an ambient temperature of 18°C.



2.3. Custom-made roller table from the workshop of the Alfred Wegener Institute, Helmholtz-Centre for Polar- und Marine Research.

### 2.2.6 Defining the culture temperature for *C. closterium* and *T. rotula.*

Microalgae were cultured at 15°C, because this temperature is in the optimal growth temperature range of the investigated microalgal species (Boyd et al., 2013). However, a temperature-controlled room at 18°C was available and facilitated large and rolling culture experiments.

Both temperatures, 15°C and 18°C were compared in a growth experiment. The diatoms *C. closterium* and *T. rotula* were inoculated with cells from mid-exponential phase in fresh medium to an initial diatom cell density of ~2000 cells/mL. The cultures were grown at 15°C and 18°C in 20 mL culture flasks (n = 4) under the regular growth conditions (Section 2.2.2).

### 2.2.7 Classification of growth phases

For all culture experiments, it was necessary to start experiments with microalgae cultures originating from the same growth phase. It is crucial that microalgae originate from the same growth phase as otherwise result are not reproducible due to different physiological performance of the microalgae.

To classify the different growth phases of the diatom *T. rotula, T. rotula* cells were grown in fresh medium starting with an initial cell density of ~2000 cells/mL. The cultures were grown at regular growth conditions (Section 2.2.2) in 20 mL culture flasks for 8 d.

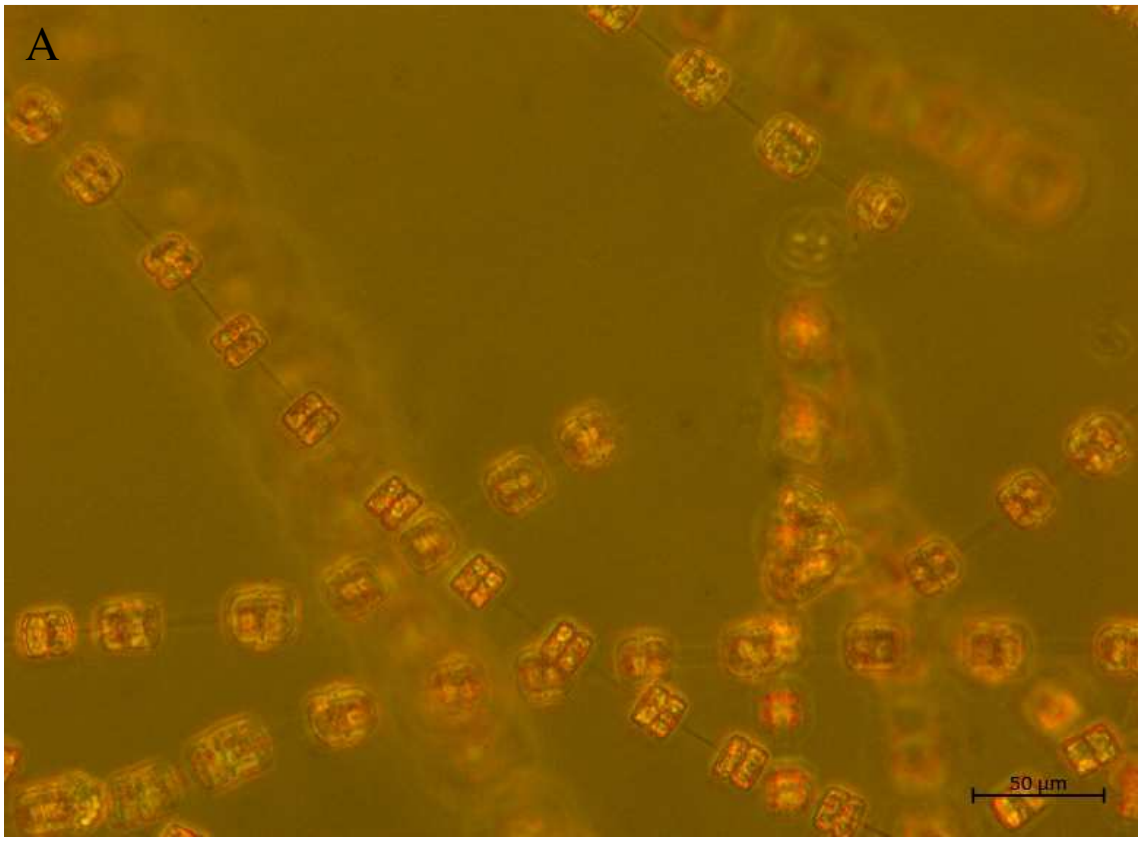
### 2.2.8 Statistical analyses

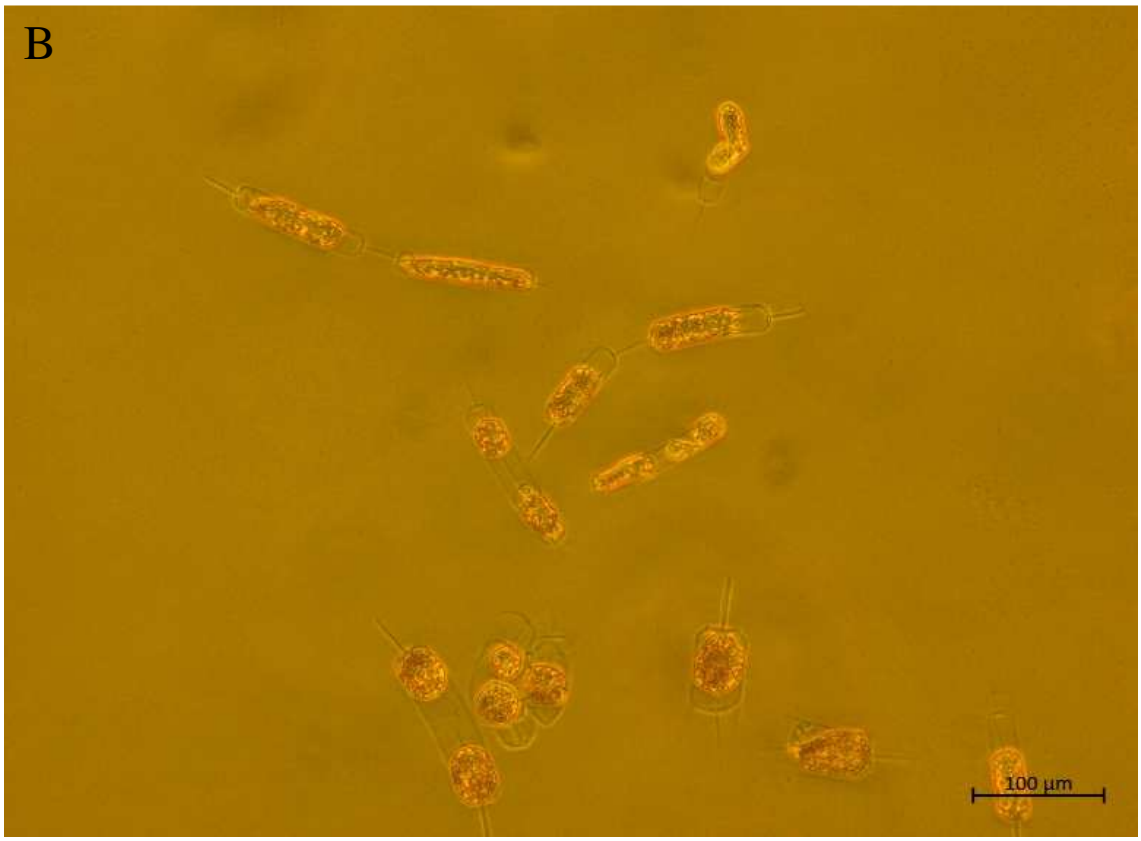
Relationships among data obtained by different growth determination methods were analysed by Pearson's product-moment correlation. Differences in algal abundance at the last measurement timepoint between the various treatments were analysed by One-way Analyses of Variance (ANOVA) followed by Tukey HSD (normally distributed data) or Kruskal Wallis One-way Analyses of Variance on Ranks followed by Dunn's test (non-normally distributed). Data were tested for normality and homogeneity of variance (Shapiro-Wilk test and Levene's-test, respectively). All statistical tests were performed at a significance level of 0.05. The analyses and plots were done using R (R Core Team, 2018) with the packages dplyr (Hadley et al., 2018), ggpubr (Kassambara, 2018), cowplot (Wilke, 2017), car (Fox and Weisberg, 2011) and ggplot2 (Wickham, 2009).

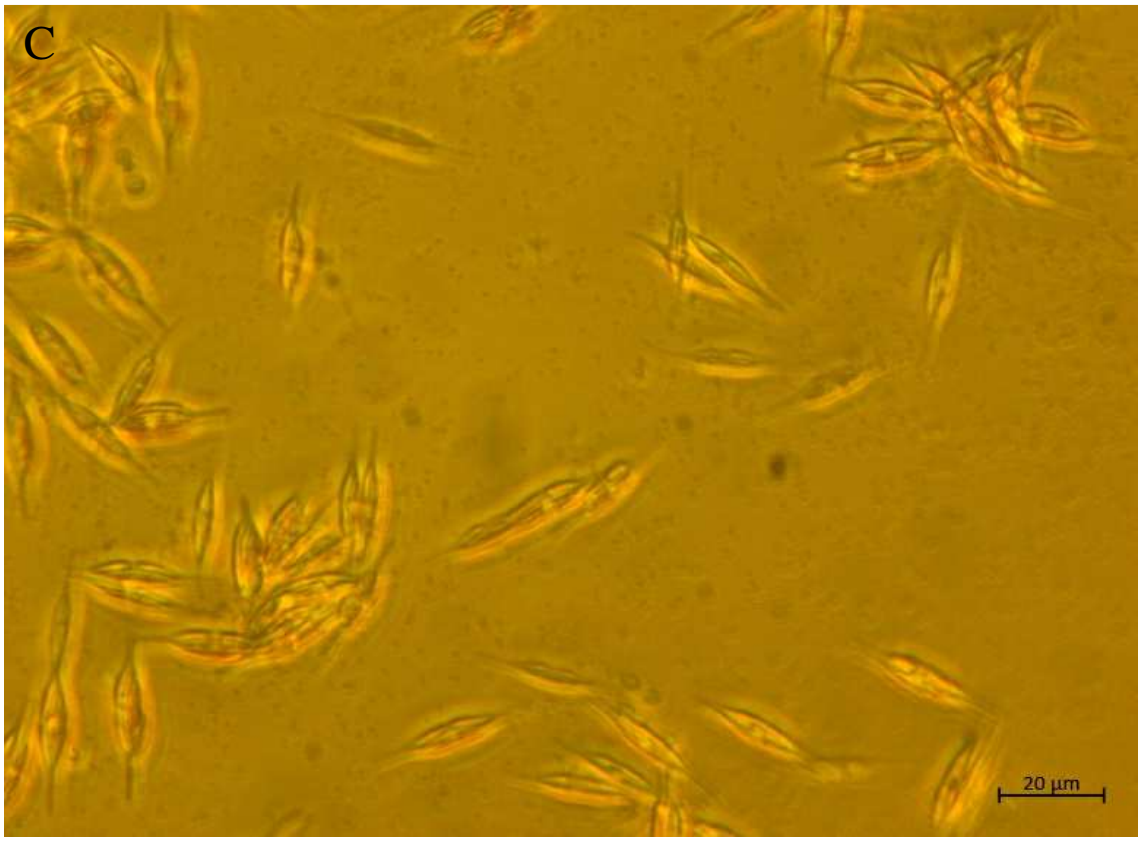
## 2.3 Results and Discussion

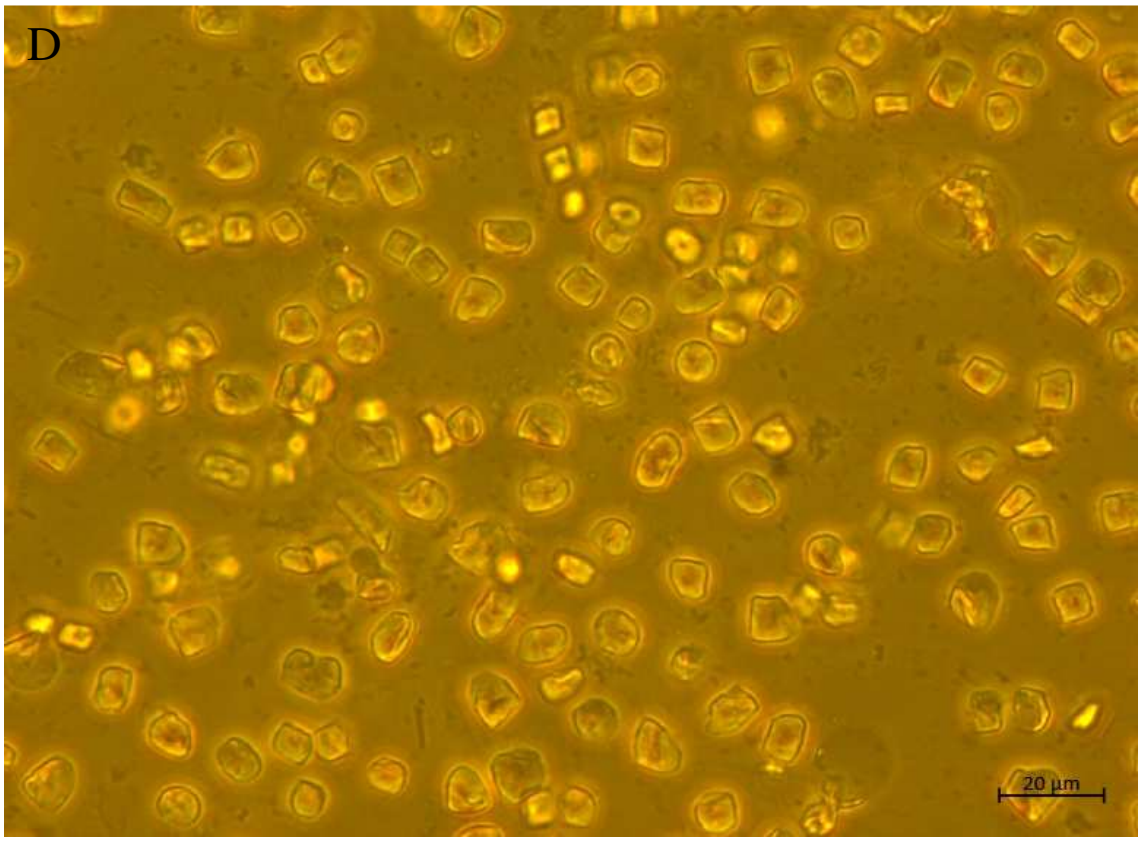
### 2.3.1 Microalgae: isolation, identification and cultivation

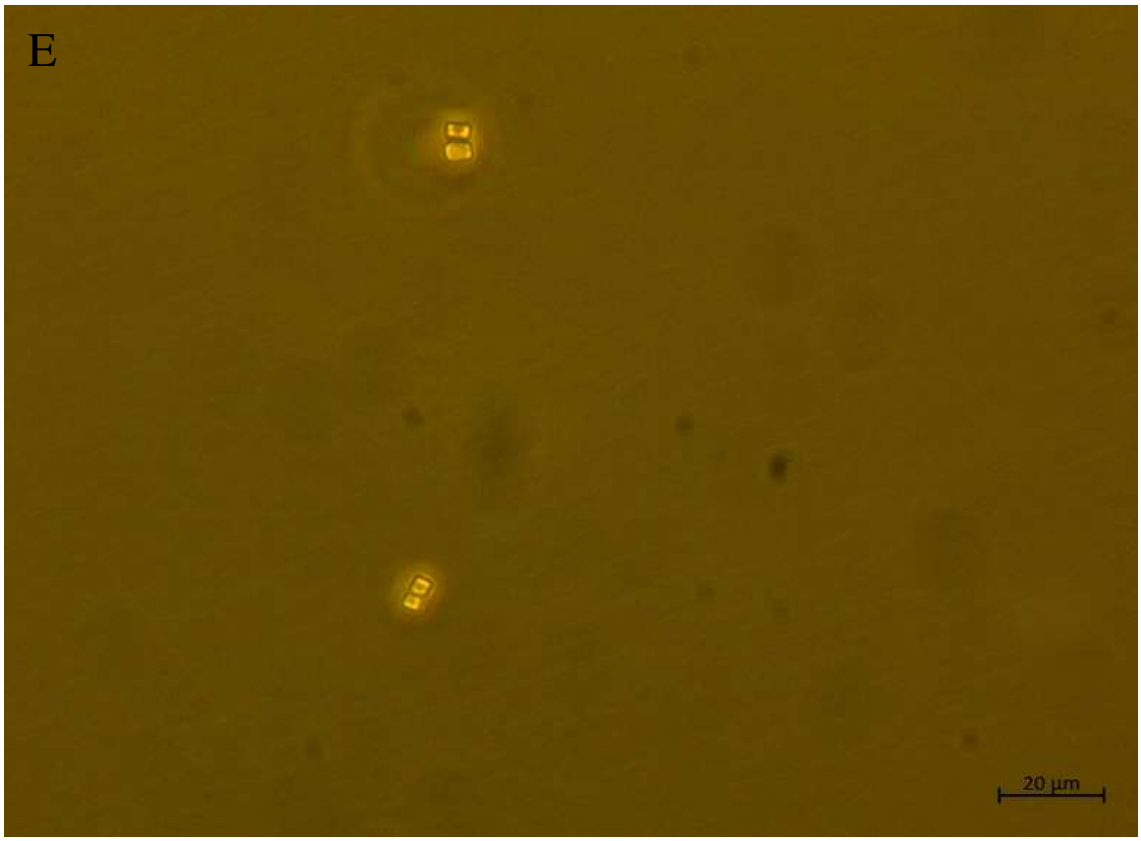
During the sampling period four diatom species belonging to four different orders were isolated (Figure 2.4). *Thalassiosira rotula* (Figure 2.4A) was isolated on 17.03.2016. *T. rotula* is a centric diatom, coin-shaped and is forming long chains, connecting single cells with a thick bundle of organic threads. *T. rotula* is a cosmopolitan species, without flagellum and is abundant during spring and fall. *Ditylum brightwellii* (Figure 2.4B) was isolated on 31.03.2016. *D. brightwellii* is a centric diatom, triangular-prism-shaped and is a mostly solitary species, sometimes forming very short chains. *D. brightwellii* is a cosmopolitan species, absent in the polar regions, without flagellum and abundant during spring. *Chaetoceros socialis* (Figure 2.4C) was isolated on 17.03.2016, 12.05.2016 and 19.05.2016. *C. socialis* is a centric diatom, elliptic-cylinder-shaped and is forming large chains with crossing adjacent spines and bundles of long spines. *C. socialis* is a cosmopolitan species with its centre of distribution in colder waters, without flagellum and is abundant from spring to autumn able to form blooms. *Cylindrotheca closterium* (Figure 2.4D) was isolated on 12.05.2016. *C. closterium* is a pennate diatom, lanceolate-shaped with long thin ends. *C. closterium* is a cosmopolitan, solitary and motile species without flagellum. *Thalassiosira pseudonana* (Figure 2.4E) and *Thalassiosira weissflogii* (Figure 2.4F) were obtained from the MARUM MPG Bridge Group Marine Glycobiology. Both are centric diatoms with a global distribution. In addition, a new *T. rotula* (A17) culture (Figure 2.4G) was isolated and cultivated in vitamin-free and vitamin containing ESAW medium on 03.09.2017 from a fresh 100 µm plankton net catch around Helgoland.

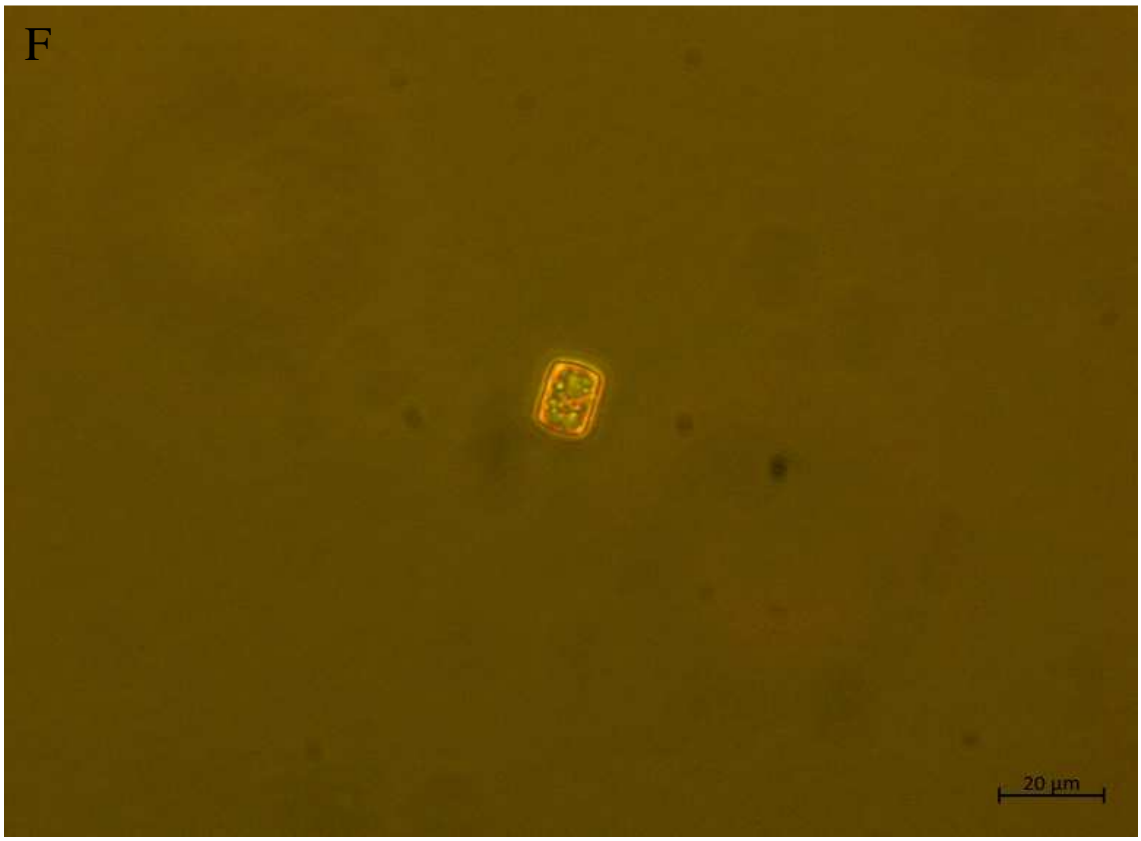

A
50 µm


B
100 µm


C
20 µm


D
20 µm


E
20 µm


F
20 µm

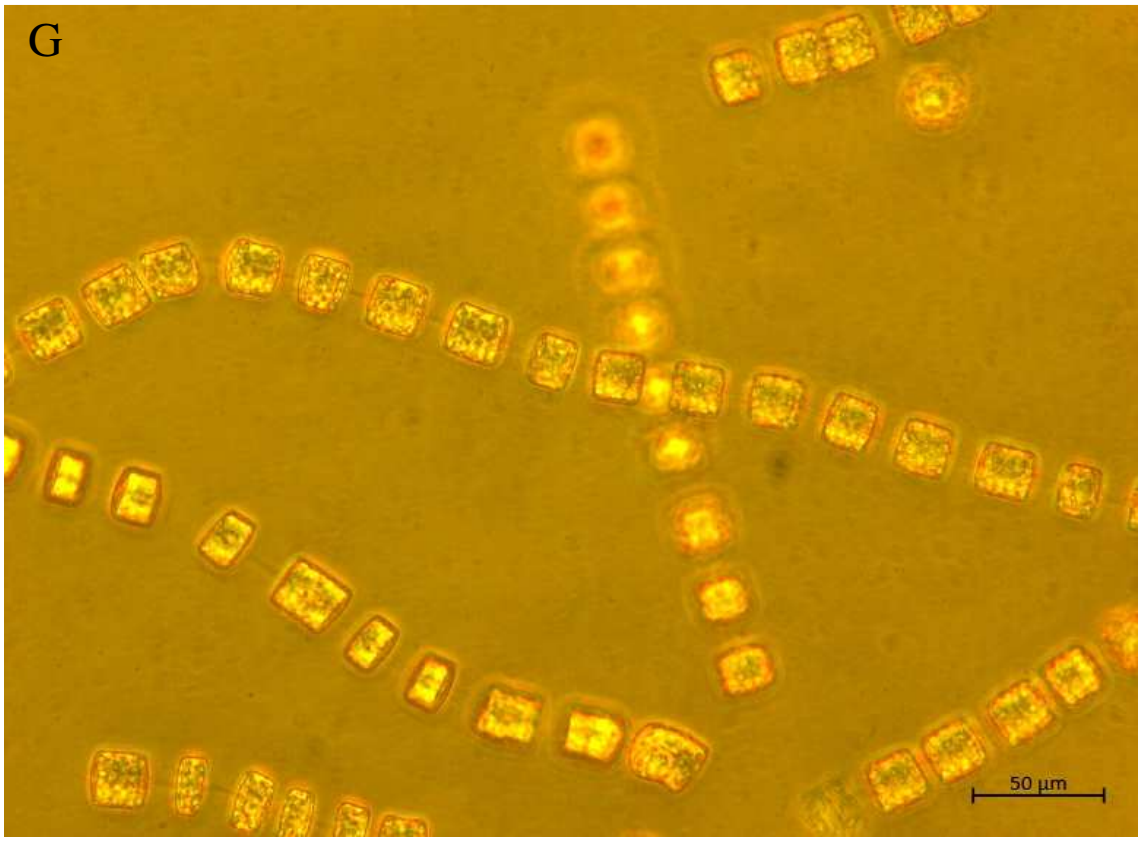
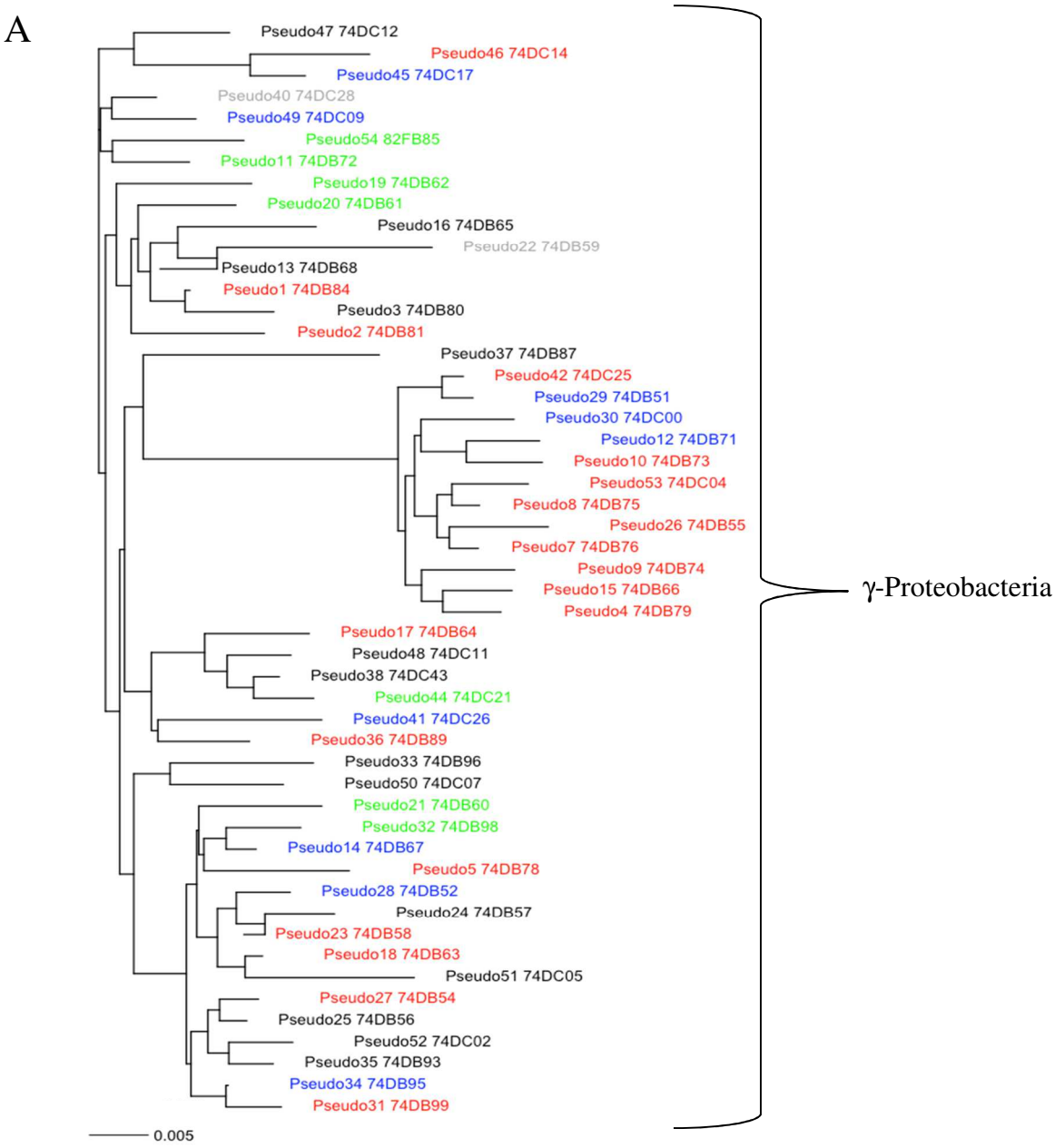



Figure 2.4. Micrographs of diatoms isolated at Helgoland or obtained from the MARUM MPG Bridge Group Marine Glycobiology. A: *Thalassiosira rotula*; B: *Ditylum brightwellii*; C: *Chaetoceros socialis*; D: *Cylindrotheca closterium*; E: *Thalassiosira pseudonana*; F: *Thalassiosira weissflogii;* G: *Thalassiosira rotula* (A17) isolated and cultivated vitamin-free. The black scale bars describe the size of the diatoms.

### 2.3.2 Bacteria: growth, isolation, and classification

200 morphologically distinguishable bacteria were isolated, of which 103 were sequenced and characterized using the Silva database (https://www.arb-silva.de). The identified bacteria belonged to the phyla Proteobacteria, Bacteriodetes, Actinobacteria and Firmicutes. Within these phyla, five different classes (Gammaproteobacteria, Alphaproteobacteria, Flavobacteriia, Actinobacteria and Bacilli) and ten different orders (Alteromonadales, Xanthomonadales, Vibrionales, Flavobacteriales, Rhodospirillales, Rhizobiales, Sphingomonadales, Actinomycedales, Bacillales and Oceanospiralles) were observed. Most of the isolated bacteria belonged to the genus *Pseudoalteromonas*, followed by the genus *Vibrio* and some other typically marine genera like *Halomonas*, *Marinobacter* and *Cobetia* (Figure 2.5).

The isolated bacteria were similar to typical taxa present in diatom blooms or in co-occurrence with diatoms (Amin et al., 2012). Many studies using culture-independent methods revealed that the bacterial communities of diatom cultures are mainly associated with bacterial phyla of Proteobacteria and Bacteroidetes (Schafer et al., 2002; Croft et al., 2005; Grossart et al., 2005; Sapp et al., 2007; Amin et al., 2015). Additional field observations showed that distinct bacterial clades of the Proteobacteria and Bacteroidetes are commonly present during phytoplankton blooms in the German Bight (Teeling et al., 2016). These observations indicate that diatoms consistently co-occur with two heterotrophic phyla Proteobacteria and Bacteroidetes.


A
Pseudo47 74DC12
Pseudo46 74DC14
Pseudo45 74DC17
Pseudo40 74DC28
Pseudo49 74DC09
Pseudo54 82FB85
Pseudo11 74DB72
Pseudo19 74DB62
Pseudo20 74DB61
Pseudo16 74DB65
Pseudo22 74DB59
Pseudo13 74DB68
Pseudo1 74DB84
Pseudo3 74DB80
Pseudo2 74DB81
Pseudo37 74DB87
Pseudo42 74DC25
Pseudo29 74DB51
Pseudo30 74DC00
Pseudo12 74DB71
Pseudo10 74DB73
Pseudo53 74DC04
Pseudo8 74DB75
Pseudo26 74DB55
Pseudo7 74DB76
Pseudo9 74DB74
Pseudo15 74DB66
Pseudo4 74DB79
Pseudo17 74DB64
Pseudo48 74DC11
Pseudo38 74DC43
Pseudo44 74DC21
Pseudo41 74DC26
Pseudo36 74DB89
Pseudo33 74DB96
Pseudo50 74DC07
Pseudo21 74DB60
Pseudo32 74DB98
Pseudo14 74DB67
Pseudo5 74DB78
Pseudo28 74DB52
Pseudo24 74DB57
Pseudo23 74DB58
Pseudo18 74DB63
Pseudo51 74DC05
Pseudo27 74DB54
Pseudo25 74DB56
Pseudo52 74DC02
Pseudo35 74DB93
Pseudo34 74DB95
Pseudo31 74DB99
0.005
γ-Proteobacteria

B

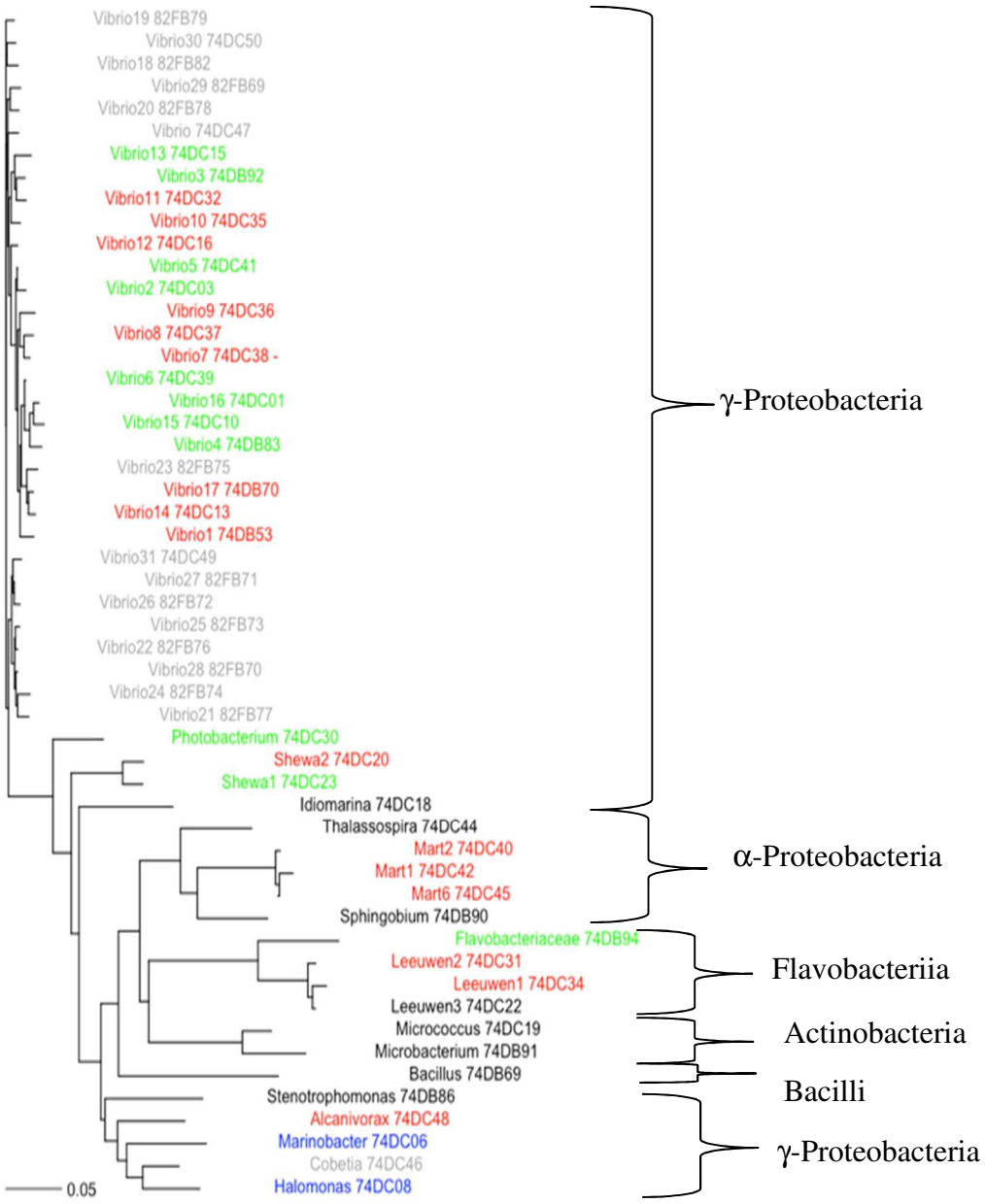



Figure 2.5. Pairwise alignment phylogenetic tree based on 16S rRNA gene sequences. A: Isolates identified as the genus Pseudoalteromonas; B: Isolates identified as diverse genera. Abbreviations: Pseudo: Pseudoalteromonas sp.; Shewa: Shewanella sp; Leeuwen: Leeuwenhoekiella sp; Mart: Martelella sp; All sequences are identified on genus level. Isolates from different sampling timepoints are shown in different colours: 17.03.16: blue; 03.09.17: grey; 05.04.16: black; 12.04.16: red; 28.04.16: green. The scale bar indicates the number of substitutions per nucleotide position. The brackets show the classification in phyla. The number behind the name describes the sequencing accession number for each sequence.

### 2.3.3 Correlation of data obtained by different growth determination methods

#### 2.3.3.1 Correlation of data acquired by a fluorescence plate reader and microalgal cell counts

To correlate growth of microalgae as determined by relative chlorophyll fluorescence and microscopic cell counts of microalgae, these data were tested by Pearson's correlation. Relative chlorophyll fluorescence was significantly correlated with microscopic cell counts (Pearson's correlation: $R = 1$; $p < 0.001$) (Figure 2.6). Therefore, relative chlorophyll fluorescence was a suitable proxy to estimate microalgal cell abundance.

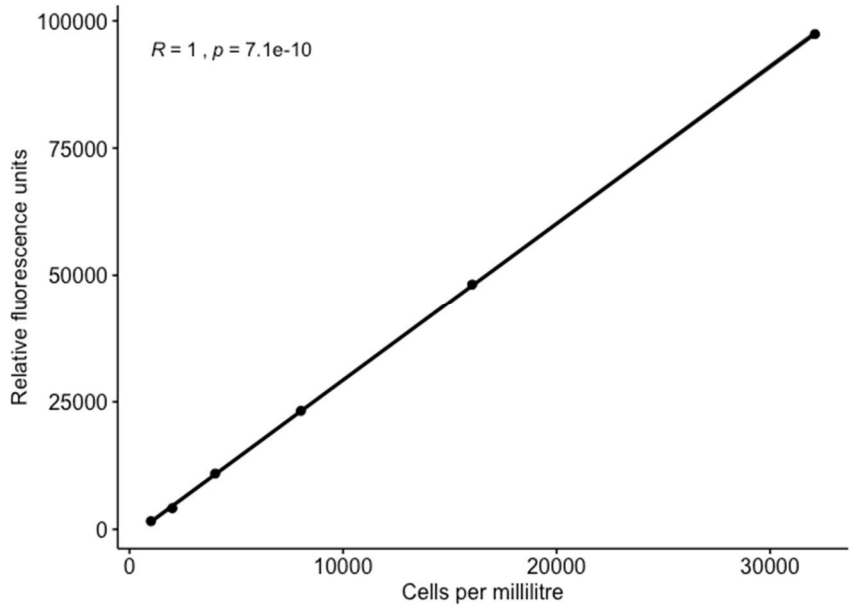



Figure 2.6 Pearson's correlation between microscopic cell counts and relative fluorescence units of *Thalassiosira rotula*. The black line represents the line of best fit. *R* and *p*-value show the calculated significance values determined by Pearson's correlation.

### 2.3.3.2 Correlation of data acquired by pulsed-amplitude-modulation fluorometry and microalgal cell counts

To correlate growth of microalgae as determined by minimal chlorophyll fluorescence and microscopic cell counts of microalgae, these data were tested by Pearson's correlation. Minimal chlorophyll fluorescence was significantly correlated with microscopic cell counts (Pearson's correlation: $R = 0.75$; $p = 0.013$) (Figure 2.7A).

The potential quantum yield ranged between 0.64 and 0.68 during the growth experiment (Figure 2.7B). This is in accordance with reported $F_v/F_m$ values of actively growing diatom cultures reported to range from 0.35 to 0.75 (Buchel and Wilhelm, 1993; Geel et al., 1997; Koblizek et al., 2001). Based on the literature, $F_v/F_m$ values above 0.6 are considered to reflect healthy and actively growing *T. rotula* cells. Determined by the significant correlation of $F_0$ and microalgae cells counts the PAM fluorometry proved to be suitable to monitor the growth of microalgae via the chlorophyll fluorescence and concurrently measure the fitness of the cells.

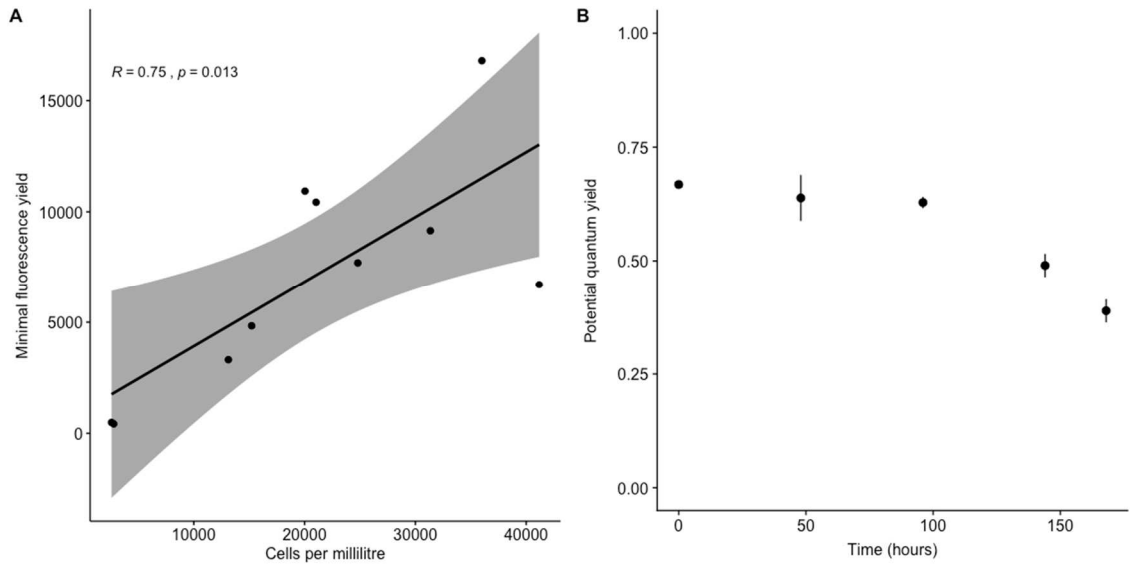



Figure 2.7. Pearson's correlation between microscopic cell counts and minimal fluorescence ($F_0$) of *Thalassiosira rotula* (A) and the corresponding potential quantum yields (B). The grey coloured area represents the 95% confidence interval and the black line shows the line of best fit. *R* and *p*-value show the calculated significance values determined by Pearson's correlation. Error bars depict ± 1 SD (n = 2).

### 2.3.3.3 Correlation of optical density data and cell counts of bacteria

To correlate abundance of bacteria as determined by OD and microscopic cell counts of bacteria, these data were tested by Pearson's correlation. OD was significantly correlated with microscopic cell counts (Pearson's correlation: $R = 0.98$; $p < 0.001$) (Figure 2.8). Therefore, OD was a suitable proxy to estimate bacterial cell abundance.

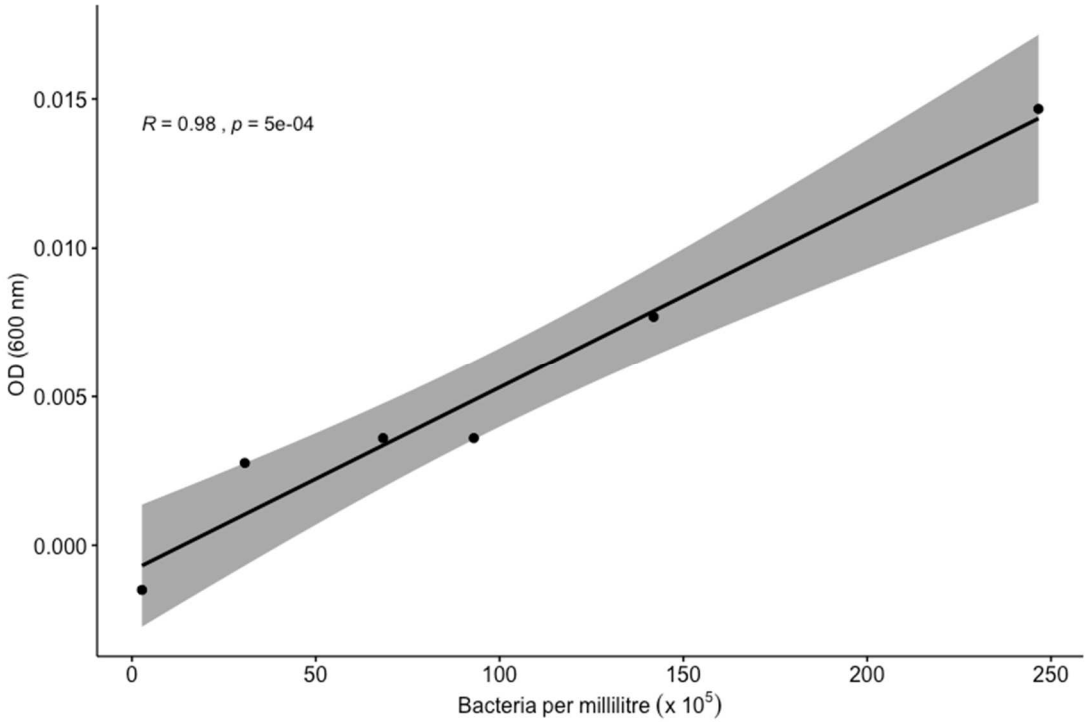



Figure 2.8. Pearson's correlation between microscopic cell counts and optical density (OD) of bacteria. The grey coloured area represents the 95% confidence interval and the black line shows the line of best fit. *R* and *p*-value show the calculated significance values determined by Pearson's correlation.

### 2.3.3.4 Correlation of cytometric and microscopic cell counts of microalgae

To correlate growth of microalgae as determined by cytometric- and microscopic cell counts of microalgae, these data were tested by Pearson's correlation. Cytometric cell counts were significantly correlated with microscopic cell counts (Pearson's correlation: $R = 1$; $p < 0.001$) (Figure 2.9). Therefore, cytometric cell counts were a suitable method to determine microalgal cell abundance.

One challenge of counting microalgae in the flow cytometer is the ability of microalgae to form chains. A conventual flow cytometer detects a chain consisting of more than one cell always as one cell. The inability to distinguish between chains and single cells restricts the function of flow cytometry to count chain-forming microalgae such as *T. rotula*. However, the established method allowed to count chain forming microalgae using the imaging function of the flow cytometer. Different chain lengths of microalgae were gated by their side scatter and chlorophyll fluorescence. The gating was verified by evaluating the images arranged in each gate and has been adjusted if necessary.

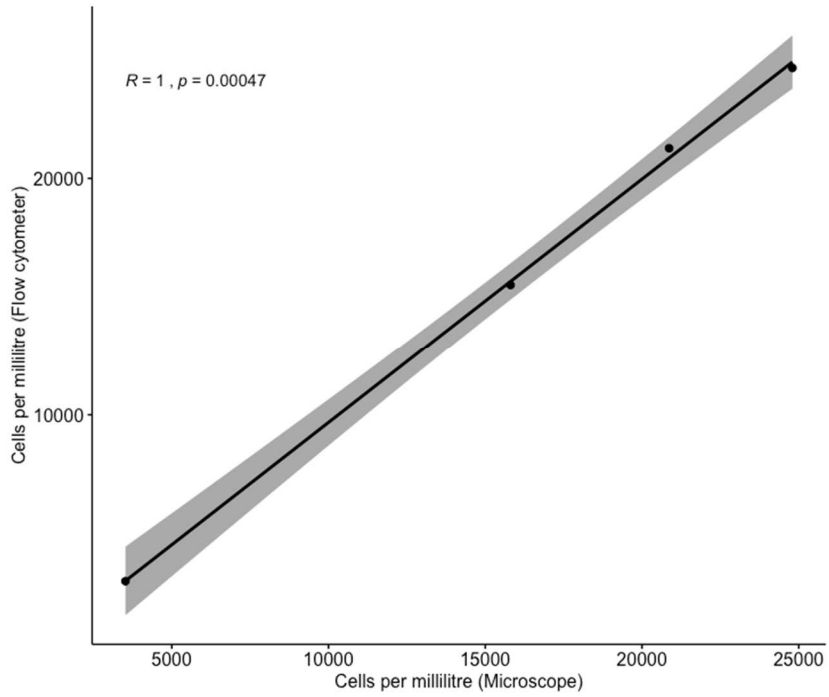



Figure 2.9. Pearson's correlation between cytometric and microscopic cell counts of the diatom *Thalassiosira rotula*. The grey coloured area represents the 95% confidence interval and the black line shows the line of best fit. R and p-value show the calculated significance values determined by Pearson's correlation.

### 2.3.4 Monitoring growth of algal cultures

#### 2.3.4.1 Comparison of media concentrations and culture volumes/vessels

To test the cultivation in 96-well plates, diatoms were cultured in 96-well plates and 20 mL cultures flasks in different media concentrations. The four diatom species grew significantly better in 96-well plates in the simply concentrated ESAW medium than in the 10x concentrated medium after 146 h (Dunn's test: *T. rotula*: $p = 0.02$; *C. socialis*: $p = 0.002$; *D. brightwellii*: $p = 0.02$; *C. closterium*: $p < 0.001$) (Figure 2.10A-D). In 20 mL culture flasks only the diatom species *D. brightwellii* grew significantly better in the simply concentrated ESAW medium than in the 10x concentrated medium after 146 h (Dunn's test: $p = 0.04$) (Figure 2.10C). The diatoms of *C. closterium*, *T. rotula* and *C. socialis* grew not significantly different in both concentrated media in 20 mL culture flasks after 146 h (Dunn's test: $p > 0.05$) (Figure 2.10A, B & D). The hypothesis that increasing the nutrient concentration would delay nutrient shortage in 96-well plates and culture flasks was rejected.

The growth of the diatoms was not significantly different in the 96-well plates and the 20 mL culture flasks after 146 h (Dunn's test: $p = 0.23$ - $0.27$) (Figure 2.10A-D), indicating that both culture containers are suitable to cultivate the investigated diatom species.

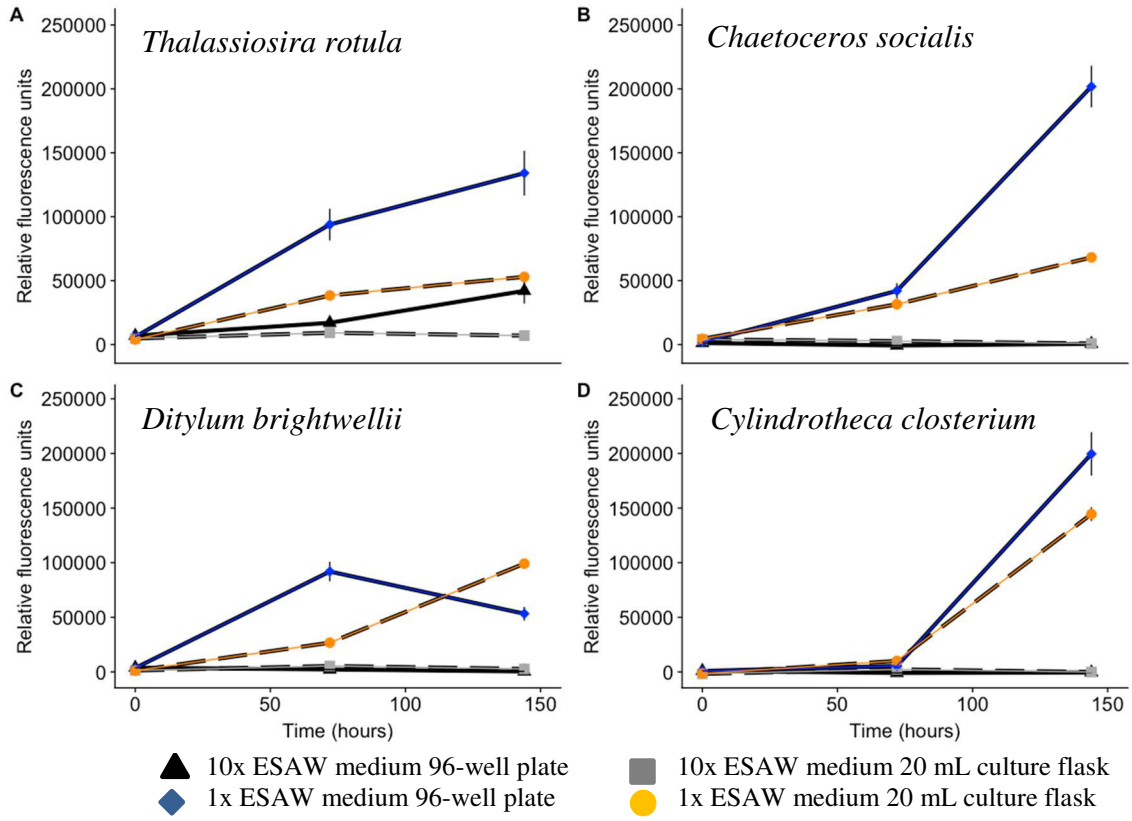



Figure 2.10. Growth of *Thalassiosira rotula* (A), *Chaetoceros socialis* (B), *Ditylum brightwellii* (C) and *Cylindrotheca closterium* (D) cultures in 1x and 10x concentrated ESAW media determined by relative fluorescence units. Culturing was conducted in black 96-well plates with clear bottom (n = 6) and 20 mL culture flasks (n = 3). Error bars depict ± 1 SD.

To verify the successful cultivation in 12-well plates, diatoms were cultured in 12-well plates and 20 mL culture flasks. The growth of the diatom *T. rotula* was not significantly different in 12-well plates and 20 mL culture flasks after 168 h (Tukey HSD: $p = 0.2$) (Figure 2.11), indicating that both 20 mL culture flasks and 12-well plates are suitable to cultivate *T. rotula*.

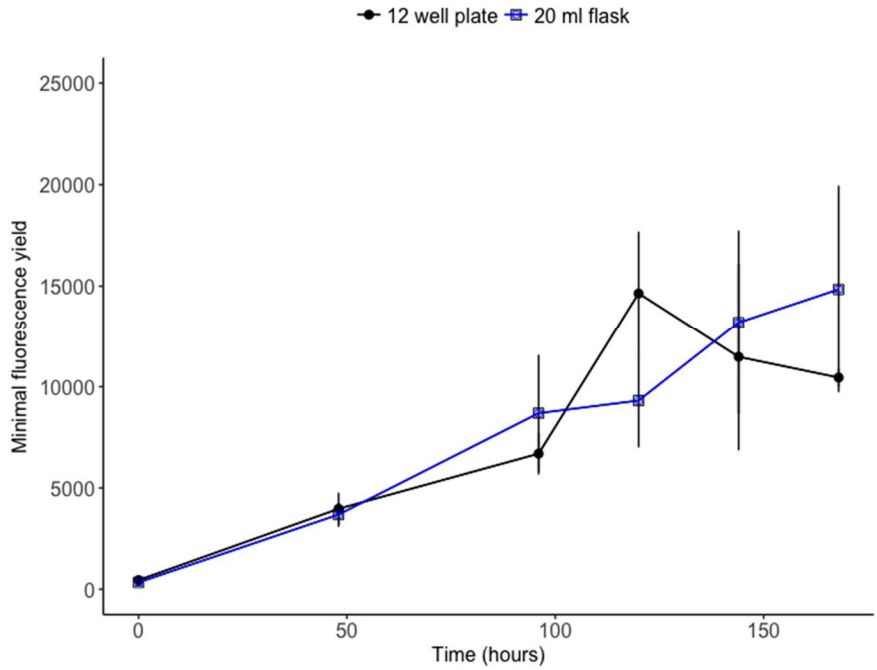



Figure 2.11. Growth of *Thalassiosira rotula* in different culturing vessels determined by the minimal fluorescence yield. *T. rotula* was cultured in 12-well plates (black) and 20 mL culture flask (blue). Error bars depict ± 1 SD (n = 3).

### 2.3.4.2 Comparison of the cultivation temperature of the diatoms *C. closterium* and *T. rotula*.

To compare the cultivation of diatoms at the temperatures 15°C and 18°C, diatoms were grown at both temperatures. The growth of the diatom *C. closterium* was significantly better at 18°C than at 15°C after 196 h (Dunn's test: $p = 0.021$) (Figure 2.12A). While the growth of *T. rotula* was same at both 15°C and 18°C after 196 h (Dunn's test: $p = 0.7728$) (Figure 2.12B) indicating that both temperatures are suitable to cultivate *T. rotula*. The increased growth of *C. closterium* due to a temperature rise is in line with the literature and was already shown for other diatoms (Montagnes and Franklin, 2001). The small temperature change from 15°C to 18°C potentially caused no change in growth for *T. rotula*, because both temperatures are in the optimal temperature range of the diatom (Boyd et al., 2013).

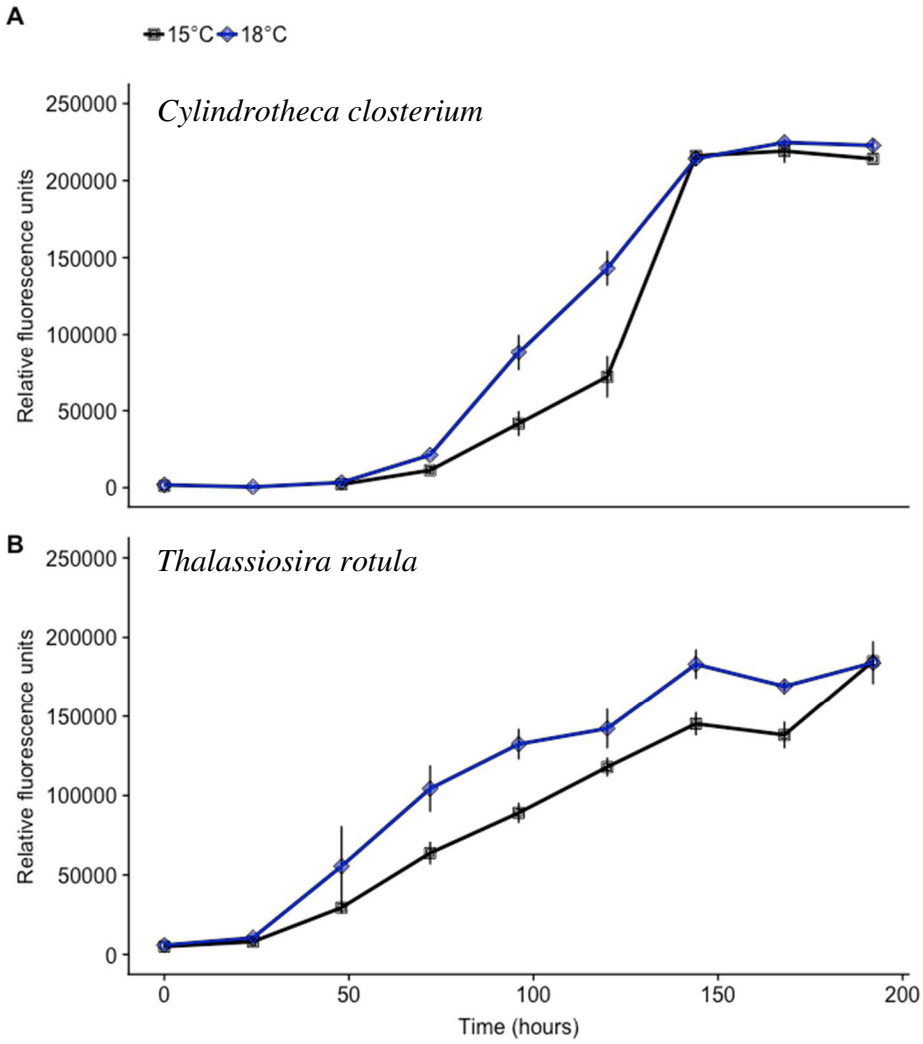



Figure 2.12. Growth of *Cylindrotheca closterium* (A) and *Thalassiosira rotula* (B) at 15°C (black square) and 18°C (blue diamond) in ESAW medium determined by relative fluorescence units. *C. closterium* and *T. rotula* were cultured in 20 mL culture flasks. Error bars depict ± 1 SD (n = 4).

### 2.3.4.3 Cultivation of *T. rotula* in 1 L glass bottles on a roller table

To test cultivation in larger volumes, diatoms were cultured in 1 L glass bottles while being rotated. The growth of *T. rotula* in 1 L glass bottles (Figure 2.13) showed comparable maxima as observed in 20 mL culture flasks at 18°C (Figure 2.12B), indicating that cultivation of *T. rotula* in 1 L glass bottles is possible.

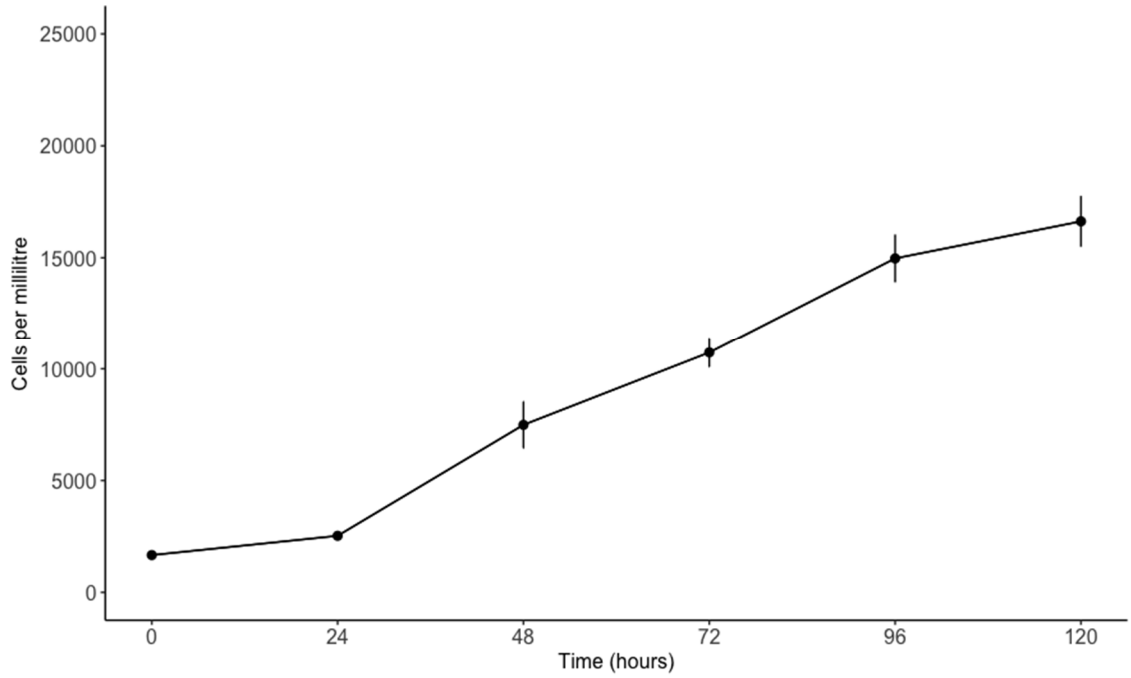



Figure 2.13. Growth of *Thalassiosira rotula* in 1 L glass bottle under regular light conditions at 18°C while being rotated on a roller table determined by microscopic cell counts. Error bars depict ± 1 SD (n = 3).

#### 2.3.4.4 Classification of growth phases

To have reproducible start conditions for cultivation, it is crucial to begin with microalgae cultures origins from the same growth phase. Therefore, the growth phases of *T. rotula* were read and classified as follows (Figure 2.14): Exponential phase 0 - 96 h; stationary phase 96 - 192 h.

The measurement of $F_v/F_m$ confirmed the growth phases, because $F_v/F_m$ of the diatoms decreases after changing into the stationary phase (Figure 2.14B). The decrease of $F_v/F_m$ is a typical sign for microalgae to switch from the exponential into the stationary phase (Wang et al., 2011; Qiu et al., 2013) and might be explained by exhaustion of some of the nutrients or accumulation of metabolic products due to the exponential growth (Sigaud-Kutner et al., 2002). To work reproducible in this thesis, all experiments were started with a culture that was between 72 and 96 h old.

Grossart et al. (2005) showed that the diatom *T. rotula* which originates from the exponential growth phase, starts to regrow again exponentially after the transfer into fresh medium, whereas the same culture originate from the stationary phase does not regrow exponentially again. Indicating that it is important to start each experiment in the same growth phase of the microalgae to obtain reproducible results.

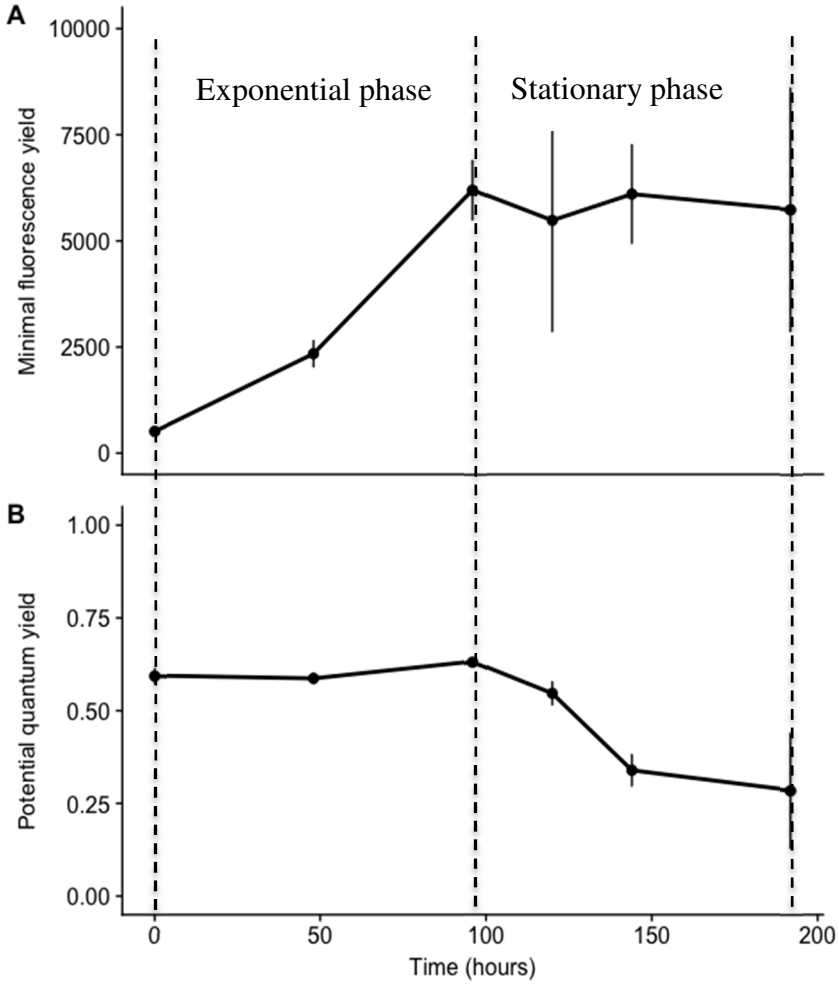



Figure 2.14. Growth of *Thalassiosira rotula* in 20 mL culture flasks determined by pulsed-amplitude-modulation fluorometry. The dotted lines indicate the exponential and stationary growth phase, respectively. A: Minimal fluorescence yield; B: Potential quantum yield. Error bars depict ± 1 SD (n = 3).

# 2.4 Supplementary information

## 2.4.1 Recipe lugol's iodine solution

- Dissolve 10 g KI in 20 mL dd$H_2O$
- Add 5 g $I_2$ (double sublimated)
- After complete solution add 50 mL dd$H_2O$ and 5 g sodium acetate

## 2.4.2 ESAW medium

Artificial seawater:

| | |
|---|---|
| NaCl | 20,8 g |
| $MgCl_2 * 6H_2O$ | 9,6 g |
| $Na_2SO_4$ | 3,5 g |
| 1M $CaCl_2$ | 9 mL |
| KCl | 0,6 g |

- Adjust the pH to 8.0 – 8.2 with 1 M HCl

Enrichment stocks:

| | |
|---|---|
| 1-5 | 1 mL/L |
| 6 | 100 µL/L |

Supplements:

I; II; III each 1 mL/L

- When everything is dissolved, fill up to 1 L
- Sterile filtrate the medium

### 2.4.2.1 Enrichment stocks

1. $NaNO_3$ 47 g/L
2. $SrCl_2 * 6H_2O$ 21 g/L
3. $Na_2SiO_3 * 9H_2O$ 30 g/L
4. $Na_2EDTA * 2H_2O$ 1.86 g
   $ZnCl_2$ 32.7 mg
   $CoCl_2 * 6H_2O$ 20.2 mg
   $Na_2MoO_4 * 2H_2O$ 126 mg
   $MnCl_2 * 4H_2O$ 475 mg

- Dissolve in 800 mL $ddH_2O$
- Adjust pH to 6 with 1 M NaOH
- Fill up to 1 L

5. $H_3BO_3$ 25 g/L
6. $Na_2SeO_3 * 5H_2O$ 1.73 g/L

### 2.4.2.2 Supplement solutions

#### Supplement I

A. $Na_2EDTA * 2H_2O$ 3.72 g

- Dissolve in 450 mL $ddH_2O$

   $FeCl_3$ 1.76 g

- Slowly add $FeCl_3$
- Adjust the pH to 6 and let the solution stir until it is clear

B. NaF 3 g
   KBr 85 g

- Dissolve in 450 mL $ddH_2O$
- Combine solution A + B
- Fill up to 1 L with $ddH_2O$

Supplement II

- Dissolve $NaHCO_3$ in a saturated solution (approximately 100 g/L)
- Add 6 g Na2-Glycerophosphate

Supplement III

| | |
|---|---|
| Thiamin HCl | 200 mg/L |
| Vitamin $B_{12}$ | 4 mg/L |
| Biotin | 2 mg/L |

## 2.4.3 Recipe marine broth / agar (1 L)

| | |
|---|---|
| Sea salt | 35 g |
| Peptone | 5 g |
| Yeast | 1 g |

For 1/10 marine broth / agar divide amount by 10

- Fill up to 1 L with dd$H_2O$
- For agar plates add 20 g/L agar
- Autoclave the solution

## 2.4.4 Minimal iron agar (1 L)

A.

| | |
|---|---|
| NaCl | 22.79 g |
| $Na_2SO_4$ | 3.98 g |
| $NaHCO_3$ | 0.031 g |
| NaF | 2.6 mg |
| $NH_4Cl$ | 0.27 g |
| $H_3BO_4$ | 27 mg |
| KBr | 83 mg |
| KCl | 0.72 g |

| | | |
|---|---|---|
| | Yeast | 0.5 g |

- Fill up to 900 mL with dd$H_2O$
- Divide into 2 x 450 ml in a 1 L bottle and add 10g Agar
- Autoclave the solution

| | | |
|---|---|---|
| B. | $MgCl_2$ | 11.8 g |
| | $CaCl_2$ | 1.46 g |
| | $SrCl_2$ | 24 mg |
| | Glucose | 3 g |
| | Ferric citrate | 0.1 g |

- Fill up to 50 mL dd$H_2O$
- Sterile filtration
- Combine 450 ml solution A with 25 ml solution B

## 2.4.5 Minimal iron EDTA agar (1 L)

| | | |
|---|---|---|
| A. | Ferric citrate | 0.1 g |
| | EDTA | 0.102 g |

- Fill up to 50 mL dd$H_2O$
- Sterile filtration

| | | |
|---|---|---|
| B. | $MgCl_2$ | 11.8 g |
| | $CaCl_2$ | 1.46 g |
| | $SrCl_2$ | 24 mg |
| | Glucose | 3 g |

- Fill up to 50 mL dd$H_2O$
- Sterile filtration

| | | |
|---|---|---|
| C. | NaCl | 22.79 g |
| | $Na_2SO_4$ | 3.98 g |
| | $NaHCO_3$ | 0.031 g |

| | |
|---|---|
| NaF | 2.6 mg |
| $NH_4Cl$ | 0.27 g |
| $H_3BO_4$ | 27 mg |
| KBr | 83 mg |
| KCl | 0.72 g |
| Yeast | 0.5 g |

- Fill up to 900 mL dd$H_2O$
- Divide into 2 x 450 mL in a 1 L-bottle and add 10 g Agar
- Autoclave the solution
- Combine 25 mL of solution A and B with 450 mL of solution C

## 2.4.6 CAS agar (200 mL)

Recipe

172 mL growth medium
1.8 g LE Agarose Biozym

- Autoclave the solution
- Add after autoclave

6 mL Casamino acids 10% (sterile filtrated)
2 mL Glucose 20% (sterile filtrated)
20 mL CAS-Fe-HDTMA Dye

Growth medium (1 L)

| | |
|---|---|
| PIPES | 30.24 g |
| $NH_4Cl$ | 1 g |
| $KH_2PO_4$ | 3 g |
| NaCl | 20 g |

- Adjust the pH to 6.8 with 10 M NaOH
- Autoclave the solution

CAS-Fe-HDTMA dye (100 mL)

A. Chromazurol S (CAS) 60.5 mg
- Dissolve in 50 mL dd$H_2O$

B. 10 mL 1 mM $FeCl_3$
- Dissolve in 100 mM HCl

C. HDTMA 72.9 mg
- Dissolve in 40 mL in dd$H_2O$

- Combine solution A and B and then with solution C
- Autoclave the solution

## 2.4.7 Lysogeny broth (LB) (1L)

| | |
|---|---|
| Yeast | 5 g |
| Tryptone | 10 g |
| NaCl | 10 g |

- Fill up to 1000 mL with dd$H_2O$
- Autoclave the solution

# Chapter 3

# 3 Development of a co-culture system to investigate interactions between microalgae and bacteria

## 3.1 Introduction

The objective of this chapter was to develop a co-culture of microalgae and bacteria to study mutualistic interactions between these organisms.

A key criterion to study microalgae-bacteria interactions are axenic microalgae. Axenic cultures are free of bacteria and therefore suitable to study the interactions of inoculated bacteria with the microalgal host. In nature, microalgae usually exist as consortia with bacteria or other microbes (Waksman, 1937; Cole, 1982). The removal of bacteria from microalgae is a multistep procedure. Several approaches have been proposed for the generation of axenic microalgae cultures, such as subcultures (Wiedeman et al., 1964), micropipette isolation (Hoshaw and Rosowski, 1973), ultrasonication (Gasulla et al., 2010), chemical treatments (Carmichael Wayne and Gorham Paul, 1974), ultraviolet radiation (Bowyer and Skerman, 1968), physical removal (Bruckner and Kroth, 2009), and antibiotic treatments (Bruckner and Kroth, 2009; Shishlyannikov et al., 2011). The application of antibiotics is the most common method for the generation and maintenance of axenic microalgae cultures. As starting an axenic cultivation with a single antibiotic has low chances to succeed, due to possible resistances of bacteria against a particular acting substance, it is recommended to use a mixture of antibiotics with different acting mechanisms. Typically, antibiotics such as Ampicillin, Gentamycin, Streptomycin, Chloramphenicol and Ciprofloxacin are used to establish axenic microalgae cultures (Shishlyannikov et al., 2011; Amin et al., 2015). Gentamycin and Streptomycin inhibit protein biosynthesis by irreversibly binding the 30S subunit of the bacterial ribosome (Vakulenko and Mobashery, 2003), while Chloramphenicol inhibits protein biosynthesis by irreversibly binding the 50S subunit of the bacterial ribosome (Kirschmann and Davis, 1969). Ampicillin inhibits cell wall synthesis of bacteria and Ciprofloxacin inhibits bacterial cell division (Rodgers et al., 1990).

To study the interactions of microalgae and bacteria, a co-culture set-up is required where all growth conditions can be adjusted in a reproducible manner. This chapter describes the preparation of axenic microalgae cultures and the development of such a co-culture system. Co-culture experiments were performed to either investigate the effect of bacterial metabolites on microalgal growth or to study the B-vitamin auxotrophy of diatoms.

## 3.2 Material and Methods

### 3.2.1 Preparation of axenic microalgae

The following materials were used unless stated otherwise: Polycarbonate membrane filters (Whatman® Nucleopore Track-Etched Membranes); a glass vacuum filter device (Sartorius); a bottle top filter holder (Thermo Scientific Nalgene).

Different experimental configurations were trialled to generate axenic diatoms (isolated and identified in Chapter 2). Three days after each exposure to antibiotics, the cultures were checked for the presence of bacteria with growth tests in liquid MB and LB and with epifluorescence microscopy after DAPI staining (Section 2.2.4.3).

#### 3.2.1.1 Antibiotics treatment and subsequent filtration (M1)

M1 was adopted from a personal communication (N. Kühne, June 7, 2016) and modified by changing the cultivation medium from K/2 to ESAW medium.

Approximately 10 mL of a diatom culture was harvested at mid-exponential phase and incubated for 120 h under regular growth conditions (Section 2.2.2) in 20 mL sterile ESAW medium containing a mixture of antibiotics (25 µg/mL Streptomycin, 33 µg/mL Gentamycin, 10 µg/mL Ciprofloxacin, 34 µg/mL Chloramphenicol, and 165 µg/mL Ampicillin) (Table 3.1). Subsequently, cells were gravity filtered onto a 0.6 µm pore-size polycarbonate membrane filter and the filter was subsequently transferred into 20 mL antibiotic-free ESAW medium.

#### 3.2.1.2 Filtration and subsequent antibiotics treatment (M2)

The application of M1 resulted in a depletion of bacteria, but did not produce axenic cultures. To improve M1, the filter pore-size was increased from 0.6 to 3 µm and the culture was filtrated and rinsed with sterile ESAW medium prior to the antibiotic treatment (Table 3.1).

#### 3.2.1.3 M2 plus detergent treatment (M3)

Applying the procedure of M2 did not lead to axenic cultures even though it resulted in a further depletion of bacteria. In order to improve M2, a detergent treatment and new concentrations of antibiotics were adopted from Shishlyannikov et al. (2011) and added to the protocol. The modifications are as follows (Table 3.1): The filter was carefully removed from the filtration device after the pre-filtration using sterile tweezers and washed for 1 min in sterile ESAW medium containing 20 µg/mL Triton-X 100 (Sigma Aldrich) detergent to remove surface-attached bacteria. Cells were re-suspended by gently shaking in sterile detergent-free ESAW medium after which the filter was discarded. Cells were again gravity filtered onto a new 3 µm pore-size polycarbonate membrane filter and rinsed with sterile ESAW medium. Afterwards, cells were washed off the filter and resuspended in sterile ESAW medium containing a similar mixture of antibiotics as above (50 µg/mL Streptomycin, 67 µg/mL Gentamycin, 20 µg/mL Ciprofloxacin, 2.2 µg/mL Chloramphenicol, and 100 µg/mL Ampicillin). After 48 h antibiotics exposure, 1 mL of the culture was transferred to antibiotic-free ESAW medium.

#### 3.2.1.4 M3 with detergent treatment inside filtration unit (M4)

The application of M3 resulted in a loss of the diatom cells during the detergent treatment. To prevent loss of cells, the detergent treatment in M4 was done inside the filtration unit.

#### 3.2.1.5 Repetition of M4 (M5)

The application of M4 removed the free-living bacteria, but some associated bacteria persisted the treatment. To achieve axenic diatom cultures, the method M4 was repeated once (Table 3.1).

Table 3.1. Methods used to prepare axenic diatom cultures described in the method sections 3.2.1.1 to 3.2.1.5. The abbreviations for the antibiotics are: A: Ampicillin; G: Gentamycin; S: Streptomycin; Ch: Chloramphenicol; Ci: Ciprofloxacin. The number describe how often the treatment was applied in the corresponding method.

| Treatment parameter | | Methods | | | | |
|---|---|---|---|---|---|---|
| | | M1 | M2 | M3 | M4 | M5 |
| | | Antibiotics treatment & subsequent filtration | Filtration & subsequent antibiotics treatment | M2 plus detergent treatment | M3 with detergent treatment (inside) | Repetition of M4 |
| Antibiotics (µg/mL) | Mixture 1 (S: 25; G: 33; Ci: 10; Ch: 34; A: 165) | 1 | 1 | | | |
| | Mixture 2 (S: 50; G: 67; Ci: 20; Ch: 2.2; A: 100) | | | 1 | 1 | 2 |
| Filtration | Pre-Antibiotics | | 1 | 1 | 1 | 2 |
| | Post-Antibiotics | 1 | | | | |
| Antibiotics exposure (h) | 48 | | | 1 | 1 | 2 |
| | 120 | 1 | 1 | | | |
| Filter pore-size (µm) | 0.6 | 1 | | | | |
| | 3 | | 1 | 1 | 1 | 2 |
| Detergent (Triton-X 100) | Outside filtration unit | | | 1 | | |
| | Inside filtration unit | | | | 1 | 2 |

### 3.2.2 Preparation of the bacterial source community as inoculum for microalgae-bacteria co-culture experiments

Non-axenic diatoms were grown in ESAW medium under regular growth conditions for 72 - 96 h (Section 2.2.2). The bacterial source communities were separated from the diatoms by gravity filtration with 0.6 or 3 µm pore-size polycarbonate membrane filters using a bottle top filter holder. The filter pore-size 0.6 µm was selected for *T. pseudonana* as the cells are < 3 µm (Hasle and Heimdal, 1970), while the pore-size 3 µm was selected for the other diatoms *T. rotula*, *T. weissflogii*, *D. brightwellii* and *C. closterium* due to the larger cells size of > 3 µm (Fryxell and Hasle, 1977; Kraberg et al., 2010). The filtrate was verified for algae contaminations (Section 2.2.4) and used for the experiments when algal-free. The bacterial source communities separated from different diatoms by filtration were used as bacterial inoculum for co-culture experiments with axenic diatoms.

### 3.2.3 Determination of the microalgae:bacteria ratio for co-culture experiments

Different ratios of microalgae and bacteria were tested to identify the ratio where growth stimulating effects for the diatom host occurred.

An axenic *T. rotula* culture was prepared according to method M5 (Section 3.2.1.5). The bacterial source community was prepared from a non-axenic *T. rotula* culture as described in section 3.2.2. Axenic diatoms from mid-exponential growing phase were inoculated with the bacterial source community in fresh ESAW medium with an initial diatom cell density of ~2000 cells/mL to achieve diatom:bacteria ratios of ~1:10, ~1:100 and ~1:1000. The co-cultures were grown for 8 d at regular growth conditions (Section 2.2.2) in 20 mL culture flasks (n = 4) (Section 2.2.5). Diatom growth was monitored by the measurement of the minimal fluorescence ($F_0$) (Section 2.2.4.1).

### 3.2.4 Examination of B-vitamin auxotrophy of the diatom *T. rotula* and vitamin synthesis from bacterial communities

As bacteria are potential producers of the vitamins $B_1$, $B_7$ and $B_{12}$, it was necessary to test for vitamin auxotrophy in axenic cultures. To test the vitamin auxotrophy status of *T. rotula*, axenic diatoms were grown in vitamin-free ESAW medium to deplete vitamins. It is hypothesized that the diatom *T. rotula* is auxotroph showing reduced growth when intra- and extracellular B-vitamins are depleted due to the absence of bacteria.

Axenic and non-axenic cultures were depleted of B-vitamins as follows. Axenic and non-axenic cultures were gravity filtered onto 3 µm pore-size polycarbonate membrane filter using a sterile glass vacuum filter device. The algal cells were rinsed 3 times with 100 mL vitamin-free ESAW medium and reduced by gravity filtration to 10 mL, transferred to 20 mL vitamin-free ESAW medium, and grown for 4 d under regular growth conditions (Section 2.2.2). The procedure was repeated twice to ensure exhaustion of intracellularly stored vitamins. Diatom growth and performance were monitored by the measurement of the minimal fluorescence ($F_0$) and potential quantum yield ($F_v/F_m$) in each growth cycle (Section 2.2.4.1).

The second objective of the repeated exhaustion of vitamins in non-axenic cultures was to increase the number of microalgae-associated bacteria in the surrounding medium that are capable to synthesize B-vitamins. The bacteria were removed repeatedly from the medium by filtration, allowing remaining algal-associated bacteria to migrate into the medium (Figure 3.1).

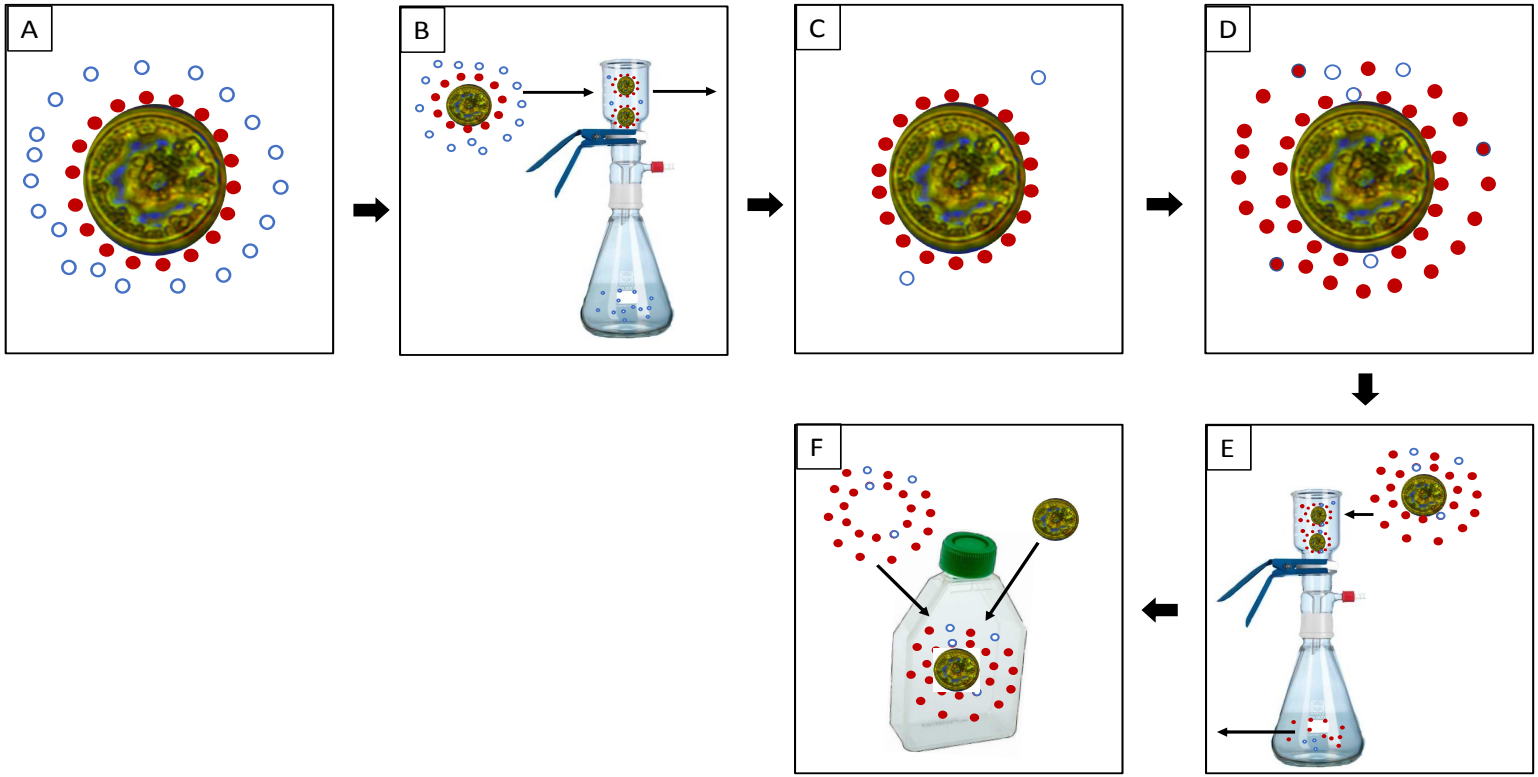



Figure 3.1. Scheme to separate free-living from associated bacteria in the surrounding medium for inoculation experiments. (A) A microalgal cell with its associated-bacterial community in red and the free-living bacterial community in blue; (B) 3 µm filtration step to reduce the number of free-living bacteria above the filter; (C) The newly created microalgae-bacteria system shortly after filtration; (D) The newly created microalgae-bacteria system after growing for 4 d; (E) Separation of the newly established bacterial source community from the microalgal cells; (F) Inoculation of the newly established bacterial source community to an axenic microalgae culture.

### 3.2.5 Effects of bacterial metabolites on diatom growth

Indole-3-acetic acid (IAA) is a plant hormone synthesized by bacteria, which is well-known to benefit growth of terrestrial plants (Won et al., 2011; Fu et al., 2015) and microalgae (Amin et al., 2015; Labeeuw et al., 2016; Segev et al., 2016). Inspired by recent findings about microalgae-bacteria interactions and IAA (Amin et al., 2015; Labeeuw et al., 2016), it was proposed to verify if IAA affected the growth of axenic and non-axenic *T. rotula* cells.

Axenic and non-axenic diatoms were inoculated from mid-exponential phase growing culture in fresh ESAW medium to an initial diatom cell density of ~2000 cells/mL (Section 2.2.4). IAA was added at four different concentrations (0.1, 1, 10 and 100 nM) to axenic and non-axenic cultures. The cultures were grown for 10 d in 20 mL culture flasks (n = 3) at regular growth conditions (Section 2.2.2). Diatom growth was monitored by the measurement of relative fluorescence (RFU) (Section 2.2.4.1).

Microalgae-bacteria interactions are suggested to either occur in the phycosphere of microalgae or in the surrounding water column (reviewed in Seymour et al. 2017). Based on the different concepts of either direct contact to the phycosphere or vertical transmission from the surrounding water, the following hypothesis was developed. It is hypothesized that bacteria have to interact with diatoms in the phycosphere to exchange nutrients (Seymour et al., 2017). To test this hypothesis, axenic *T. rotula* cells were either inoculated with a filtrate containing bacterial exudates plus bacteria (3 µm) or simply with bacterial exudates (0.2 µm). With this experimental setup it was furthermore tested whether bacteria only support microalgae growth when attached to the diatom phycosphere or also via dissolved exudates by vertical transmission from the surrounding water column.

Axenic (Section 3.2.1.5) and non-axenic cultures of *T. rotula* were inoculated from mid-exponential phase growing culture in fresh medium to an initial diatom cell density of ~2000 cells/mL (Section 2.2.4). The bacterial source community was prepared as described in section 3.2.2. Bacterial exudates were prepared as follows: A mid

exponential phase growing diatom culture was vacuum filtrated onto 0.2 µm pore-size polycarbonate membrane filter using a bottle top filter holder. The filtrate was added to the culture in the same ratio as it was prepared for the bacterial source community. Both inocula were added to the axenic *T. rotula* cultures in a quantity to achieve a ~1:100 diatom:bacteria ratio (Section 3.2.3). The co-cultures were grown for 7 d in 20 mL culture flasks (n = 3) under regular growth conditions (Section 2.2.2). Diatom growth was monitored by the measurement of relative fluorescence (RFU) (Section 2.2.4.1).

### 3.2.6 Statistical analysis

Differences in algal abundance at the last measurement timepoint between the various treatments were analysed by One-way Analyses of Variance (ANOVA) followed by Tukey HSD (normally distributed data) or Kruskal Wallis One-way Analyses of Variance on Ranks followed by Dunn's test (non-normally distributed). Data were tested for normality (Shapiro-Wilk test) and homogeneity (Levene's-test) of variance. All statistical tests were performed at a significance level of 0.05. The analyses and plots were done using R (R Core Team, 2018) with the packages dplyr (Hadley et al., 2018), ggpubr (Kassambara, 2018), cowplot (Wilke, 2017), car (Fox and Weisberg, 2011) and ggplot2 (Wickham, 2009).

## 3.3 Results and Discussion

### 3.3.1 Preparation of axenic diatom cultures

Axenicity of microalgae cultures was verified once no bacteria were detected in the culture under the fluorescence microscope after DAPI staining and no bacteria grew in liquid MB and LB media after inoculation with the microalgal culture.

The final treatment to prepare axenic microalgae consisted of a 3 µm filtration and a detergent treatment to remove free-living and associated bacteria prior to the antibiotics treatment (Table 3.2). The antibiotics treatment was performed with the second described

mixture for 48 h (Table 3.2). The complete procedure was repeated once to achieve axenicity (Table 3.2).

The first hypothesis under investigation was that the diatoms *T. rotula*, *D. brightwellii*, *C. closterium* and *C. socialis* can be rendered axenic. Based on the results of the preparation of axenic diatom cultures, the hypothesis was accepted for *T. rotula* and rejected for *D. brightwellii*, *C. closterium* and *C. socialis* (Table 3.2).

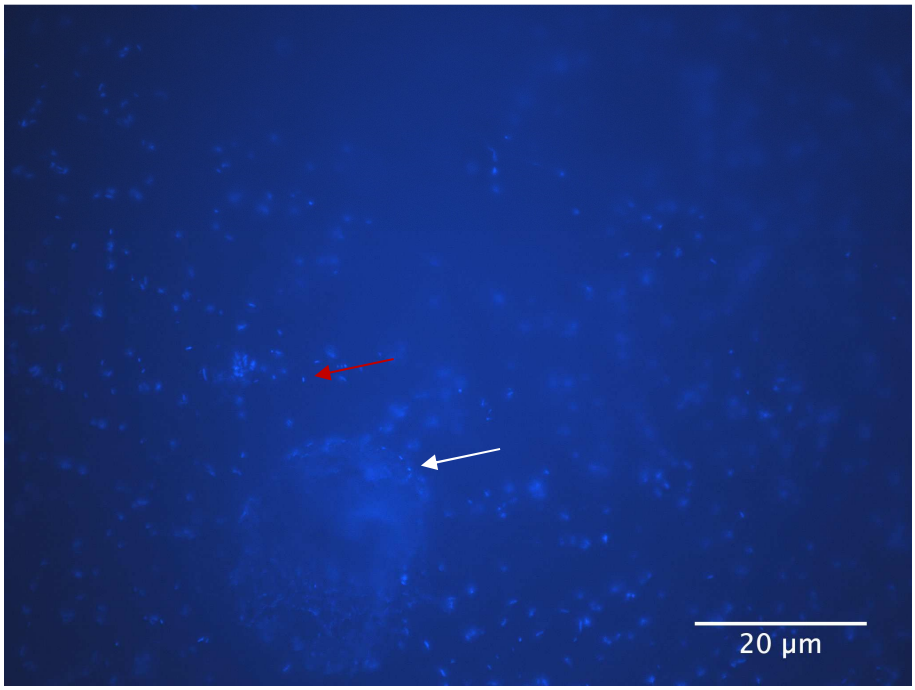



Figure 3.2. Antibiotic treated *Thalassiosira rotula* cells after the treatment according to method 1 (Section 3.2.1.1). The picture was taken after growing for 72 h in antibiotic-free ESAW medium. The white arrow points to associated and the red arrow to free-living bacteria. Scale bar: 20 µm.

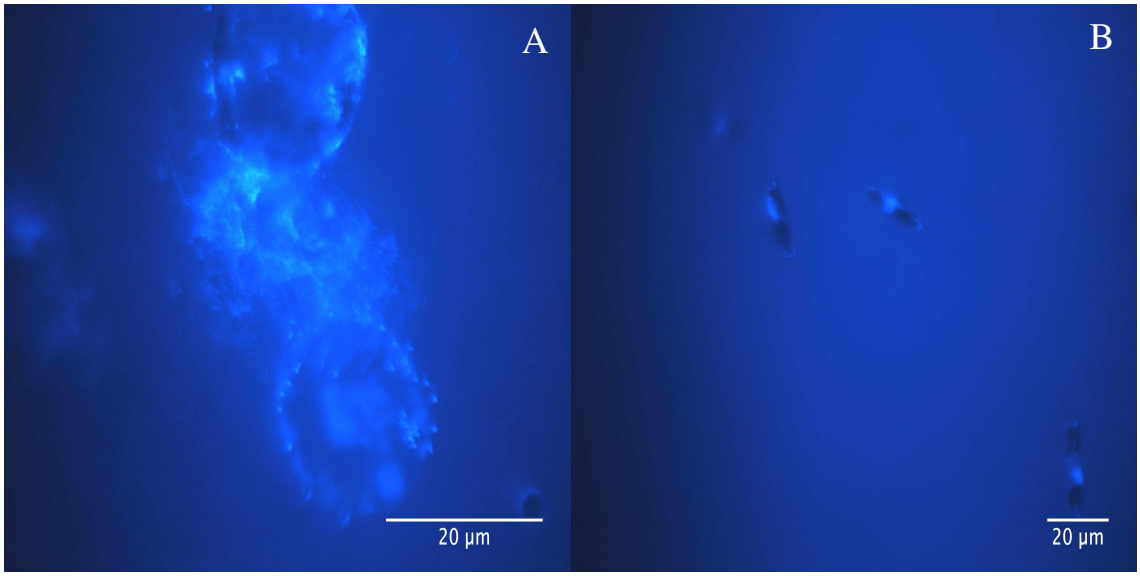



Figure 3.3. Antibiotic treated *Thalassiosira rotula* (A) and *Cylindrotheca closterium* (B) cells after the treatment according to method 2 (Section 3.2.1.2). The pictures were taken after growing for 72 h in antibiotic-free ESAW medium. Scale bar: 20 µm.

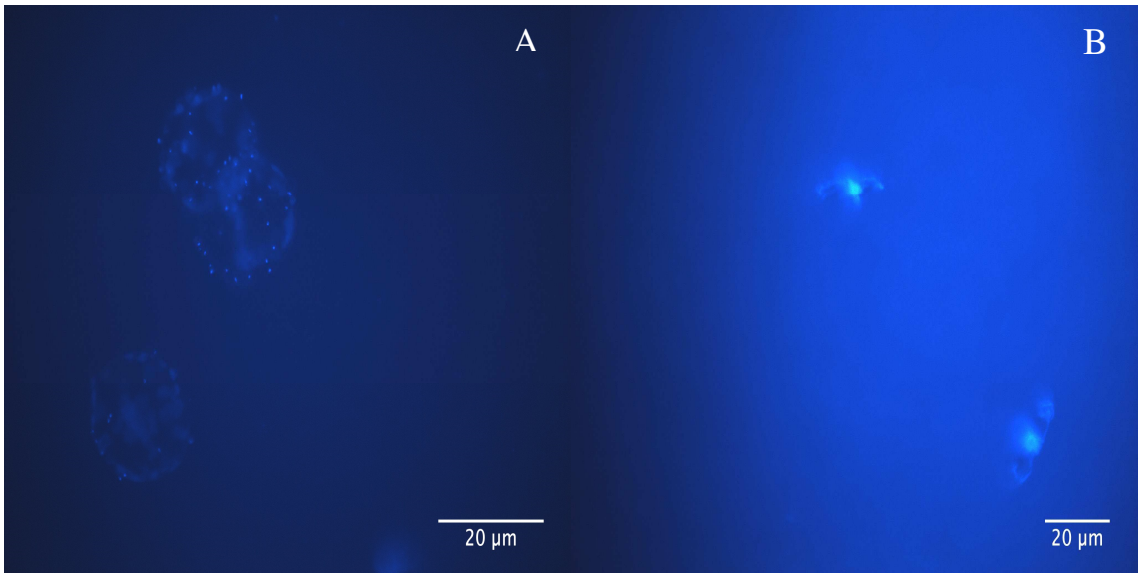



Figure 3.4. Antibiotic treated *Thalassiosira rotula* (A) and *Cylindrotheca closterium* (B) cells after the treatment according to method 4 (Section 3.2.1.4). The pictures were taken after growing for 72 h in antibiotic-free ESAW medium. Scale bar: 20 µm.

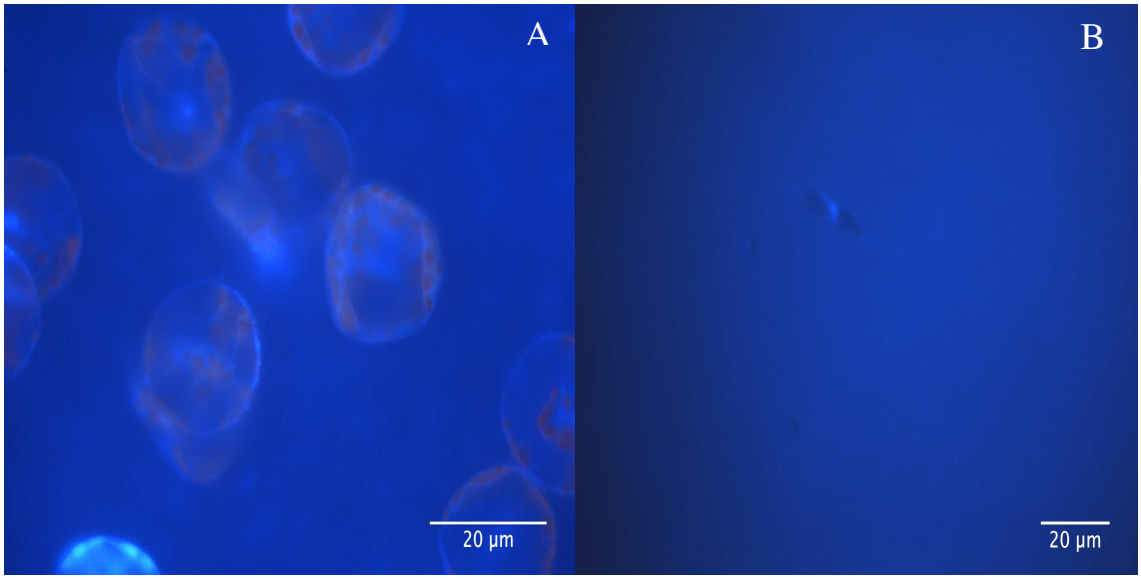



Figure 3.5. Antibiotic treated *Thalassiosira rotula* (A) and *Cylindrotheca closterium* (B) cells after the treatment according to method 5 (Section 3.2.1.5). The pictures were taken after growing for 72 h in antibiotic-free ESAW medium. Scale bar: 20 µm.

Table 3.2. Results of the preparation of axenic diatom cultures. The plus (+) denotes a reduction while a minus (-) indicates no reduction of bacterial numbers or a damage of the cells. A double plus (++) denotes an axenic microalgae culture.

| **Method** | **Adopted from** | **Treatment parameter** | **Decreased number of bacteria in diatom culture** | | | | **Results of axenicity** |
|---|---|---|---|---|---|---|---|
| | | | ***Thalassiosira rotula*** | ***Ditylum brightwellii*** | ***Cylindrotheca closterium*** | ***Chaetoceros socialis*** | |
| M1 | N. Kühne, personal communication 2016 | Antibiotics mixture 1<br>0.6 µm post-filtration | + | - | + | + | Figure 3.2 |
| M2 | N. Kühne, personal communication 2016 | 3 µm pre-filtration<br>Antibiotics mixture 1 | + | - | + | - | Figure 3.3 |
| M3 | Shishlyannikov et al., 2011 | 3 µm pre-filtration<br>Antibiotics mixture 2<br>Triton-X 100 (Outside filtration unit) | + | | + | | |
| M4 | Shishlyannikov et al., 2011 | 3 µm pre-filtration<br>Antibiotics mixture 2<br>Triton-X 100 (Inside filtration unit) | + | | + | | Figure 3.4 |
| M5 | Shishlyannikov et al., 2011 | Twice M4 | ++ | | + | | Figure 3.5 |

### 3.3.2 Growth comparison of axenic cultures

The growth of the *T. rotula* cultures before and after the application of method M4 and M5 were compared. The *T. rotula* culture before (Dunn's test: $p = 0.002$) and after application of method M4 (Dunn's test: $p = 0.041$) grew significantly better than the culture treated with method M5 after 196 h (Figure 3.6). The growth decline of the axenic culture originated from method M5 (Section 3.2.1.5) suggests that the algal cells lack essential nutrients, which were produced by bacteria under non-axenic conditions. This pattern is supported by frequent observations that microalgal growth declines in the absence of bacteria under limiting or non-limiting nutrient conditions (Bolch et al., 2011; Windler et al., 2014; Bolch et al., 2017). The results confirm that the treatment performed in method M4 did not eradicate all bacteria, because the growth was not significantly different compared to the non-treated culture after 196 h (Dunn's test: $p > 0.05$) and indicated that the essential nutrients are still present. The fluorescence microscopy of the *T. rotula* culture derived from method M4 showed that associated-bacteria persisted the treatment according to method M4 (Figure 3.4A). Therefore, the essential nutrients were most likely supplied from the few remaining bacteria in the phycosphere of the *T. rotula* culture derived from method M4. The strong resistance of the attached bacteria in this culture suggests that the microalga protects these bacteria by the production of large amounts of extracellular polymeric substances (Bruckner et al., 2008; Debenest et al., 2009). The microalgae host protects the associated bacteria because of the essential support function for microalgae growth and performance, like the production of B-vitamins (Croft et al., 2006; Cruz-Lopez and Maske, 2016).

The second hypothesis under investigation was that diatoms cannot grow axenically over multiple generations in ASW medium. The second hypothesis was accepted for *T. rotula*, as the axenic culture did not grow without bacteria in ASW medium (Figure 3.6).

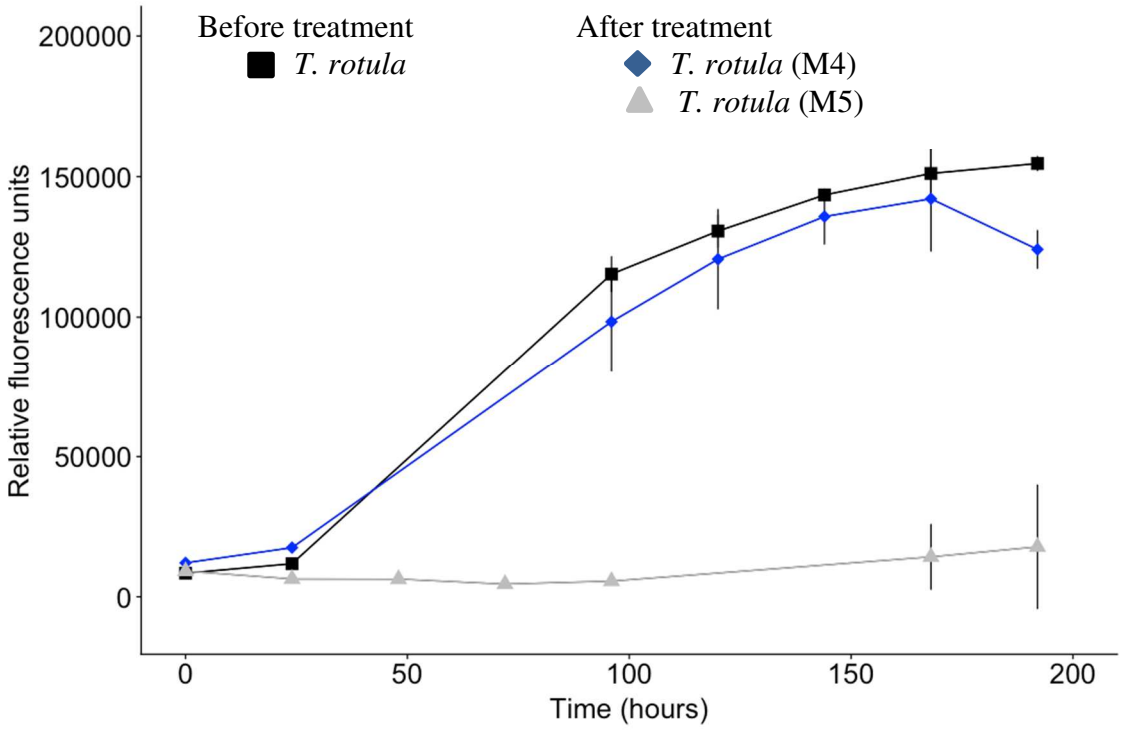



Figure 3.6. Growth of *Thalassiosira rotula* cultures treated with methods M4 and M5 to prepare axenic diatom cultures. Growth of *T. rotula* cells originated from M4 (Blue diamond) and M5 (Grey triangle) with the corresponding *T. rotula* culture before the treatments (Black square) The growth of the axenic culture derived from method M5 was significantly different to the origin culture before the method M4 and to the culture after method M4. Growth was monitored by relative fluorescence units (RFU) (n = 3). Error bars depict ± 1 SD.

### 3.3.3 Preparation of the bacterial source communities as inoculum for microalgae–bacteria co-culture experiments

To verify whether the separation of the bacterial source community from the diatom cultures was successful, the bacterial source communities were investigated by microscopy of DAPI-stained cells. The inspection of present cells in the filtrates revealed no contaminations with diatoms (Figure 3.7 red arrow indicates bacteria). The proved separation of bacteria and diatoms (Figure 3.7) showed that the used filter pore-sizes were suitable for the different diatom species. Therefore, the separated bacterial source communities of the five investigated diatom species were suitable as bacterial inocula.

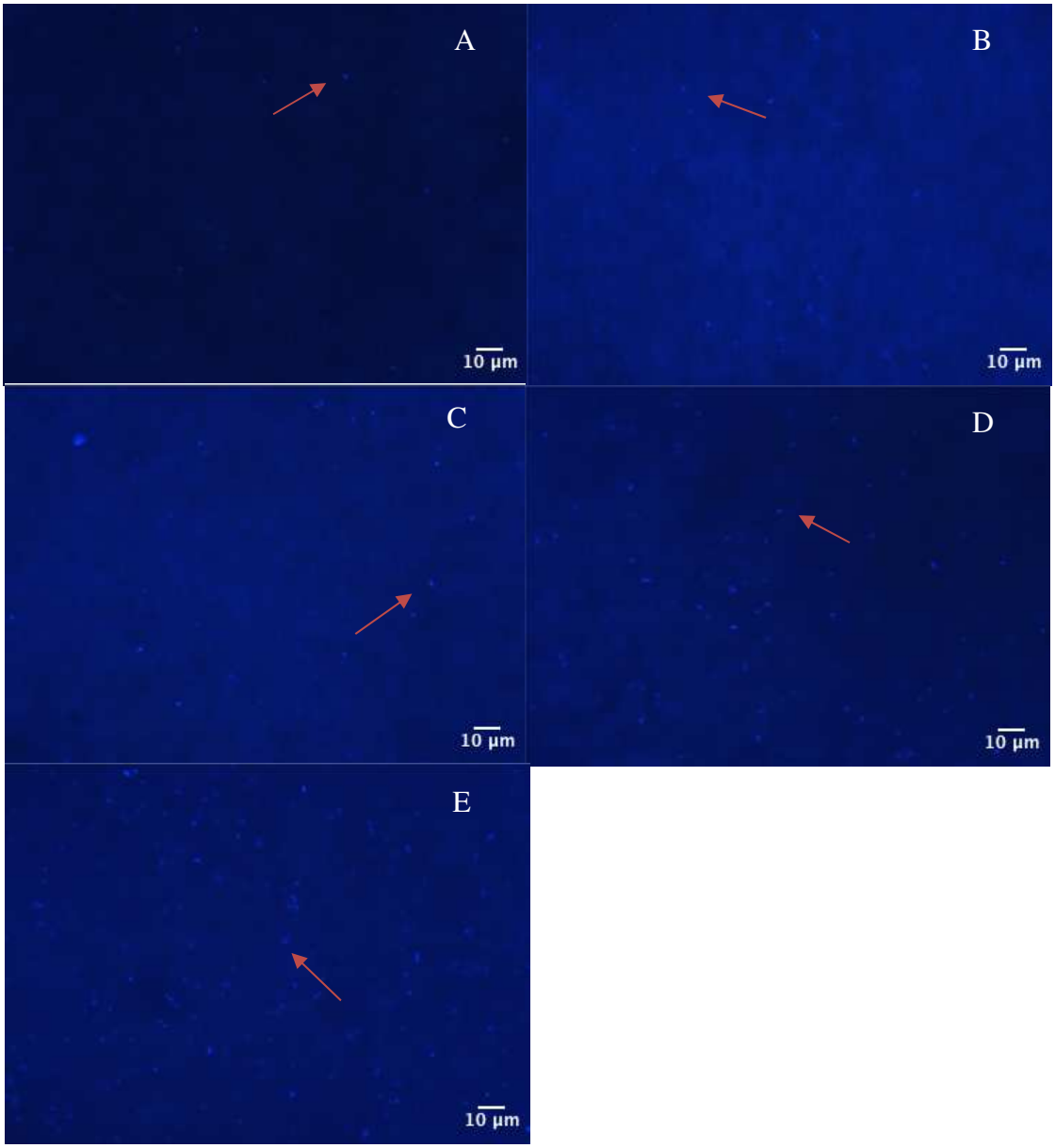



Figure 3.7. Epifluorescence micrographs of DAPI stained bacterial source communities obtained from different diatom hosts via 0.6 or 3 µm pore-size gravity filtration. The diatom culture of *Thalassiosira pseudonana* (A) was filtered through a 0.6 µm membrane filter. The diatoms *Thalassiosira weissflogii* (B), *Cylindrotheca closterium* (C), *Thalassiosira rotula* (D) and *Ditylum brightwellii* (E) were filtered through 3 µm membrane filters. The red arrows show bacteria stained with DAPI.

### 3.3.4 Effect of microalgae:bacteria ratio on the growth of *T. rotula*

The potential growth effect on the axenic diatom *T. rotula* was examined by the inoculation of different ratios of diatoms to bacteria. The growth of the axenic *T. rotula* was not significantly affected by the inoculation of a bacteria to diatoms ratio of 1000:1 after 196 h (Dunn's test: $p > 0.05$) (Figure 3.8). However, the inoculation of bacteria to diatoms ratios of 10:1 (KW: $p = 0.0018$) and 100:1 (Dunn's test: $p = 0.0444$) affected the growth of the axenic *T. rotula* significantly after 196 h (Figure 3.8). Based on the positive growth effect of the inoculation of a ratio of bacteria to diatoms 100:1 and the regularly observed ratio of bacteria to diatoms 100:1 in non-axenic *T. rotula* cultures in the laboratory, the ratio of bacteria to diatoms 100:1 was selected for further experiments. Furthermore, the chosen ratio is supported by similar observations of bacteria and microalgae densities in the ocean (Whitman et al., 1998; Seymour et al., 2017).

The third hypothesis under investigation was that diatoms and bacteria exchange nutrients. The hypothesis was accepted, because the culture started to regrow after the axenic culture was inoculated with bacteria (Figure 3.8).

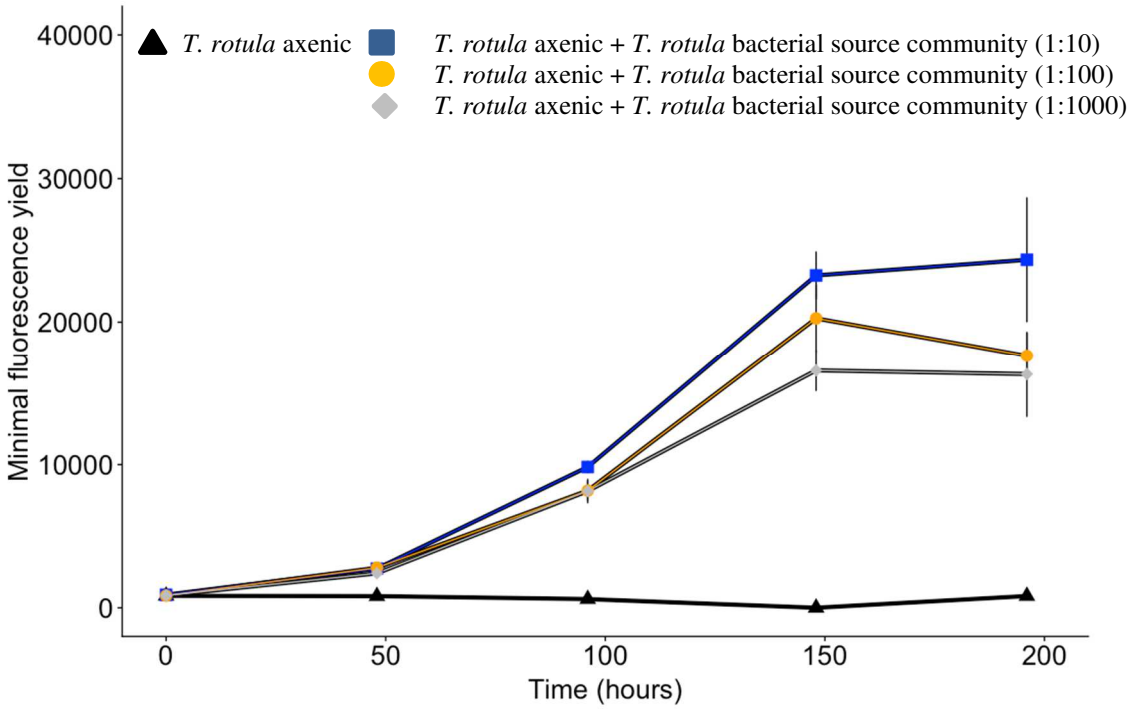



Figure 3.8. Growth of axenic *Thalassiosira rotula* co-cultures with the original bacterial source community in different ratios of diatoms to bacteria. Growth of *T. rotula* axenic, axenic + *T. rotula* bacterial source community (1:10), axenic + *T. rotula* bacterial source community (1:100), axenic + *T. rotula* bacterial source community (1:1000). The growth of an axenic *T. rotula* culture was significantly affected by the inoculation of its own bacterial source community in the diatom:bacteria ratio 1:10 and 1:100 after 196 h. Algal growth was monitored by minimal fluorescence ($F_0$) (n = 3). Error bars depict ± 1 SD.

### 3.3.5 Examination of B-vitamin auxotrophy of the diatom *T. rotula* and vitamin synthesis from bacterial communities

In section 3.3.4 it was described that the axenic culture of *T. rotula* did not grow after removal of bacteria (Figure 3.8). Therefore, a new axenic culture needed to be generated before each experiment, because the culture could not be cultivated over several generations in ASW medium. New axenic cultures were prepared for the experiments described in the sections 3.3.2, 3.3.4 and 3.3.6.

At some point of this thesis, the newly established axenic culture grew although the bacteria were removed successfully, caused by a relocation of the diatom cultures to the lab in Canada. To confirm the axenicity, the culture was verified several times with the described methods in section 3.2.1 and additionally with flow cytometry (Section 2.2.4.4). The axenicity was confirmed multiple times and the culture was used for experiments. Since then, the axenic *T. rotula* was cultivated in vitamin containing ESAW medium over several generations. Thus, it was possible to use the same culture in all subsequent experiments.

The B-vitamin auxotrophy of the diatom *T. rotula* was examined by depleting vitamins across repeated growth cycles. The growth of the axenic *T. rotula* culture declined after two consecutively growth cycles of 72 - 96 h in vitamin-free ESAW medium (Figure 3.9A). After 264 h (11 d) the growth of the axenic *T. rotula* culture was significantly different to the non-axenic control (Dunn's test: $p = 0.0072$) (Figure 3.9A). Similar to $F_0$, $F_v/F_m$ of the axenic culture decreased at the end of the second growth cycle, while $F_v/F_m$ of the non-axenic culture did not change. Within the last growth cycle (168 - 264 h), $F_v/F_m$ decreased to 0.2 (Figure 3.9B). $F_v/F_m$ below the threshold of 0.35 is a sign for perishing or resting stage cells (Buchel and Wilhelm, 1993; Geel et al., 1997; Koblizek et al., 2001), here indicating that the axenic *T. rotula* cells were scarce of vitamins for growth.

Consequently, the decrease in $F_0$ and $F_v/F_m$ showed that the axenic *T. rotula* culture was depleted of B-vitamins during the last growth cycle. Because only the B-vitamins biotin ($B_7$), cobalamin ($B_{12}$) and thiamine ($B_1$) were removed from the ESAW medium, *T. rotula* is auxotroph for these three B-vitamins (Figure 3.9).

The hypothesis under investigation was that the diatom *T. rotula* was B-vitamin auxotroph and declined in growth when intra- and extracellular vitamins were depleted. The axenic *T. rotula* culture declined in growth after 144 h cultivation in vitamin-free ESAW medium and did not regrow afterwards (Figure 3.9). Hence, the fourth hypothesis was accepted.

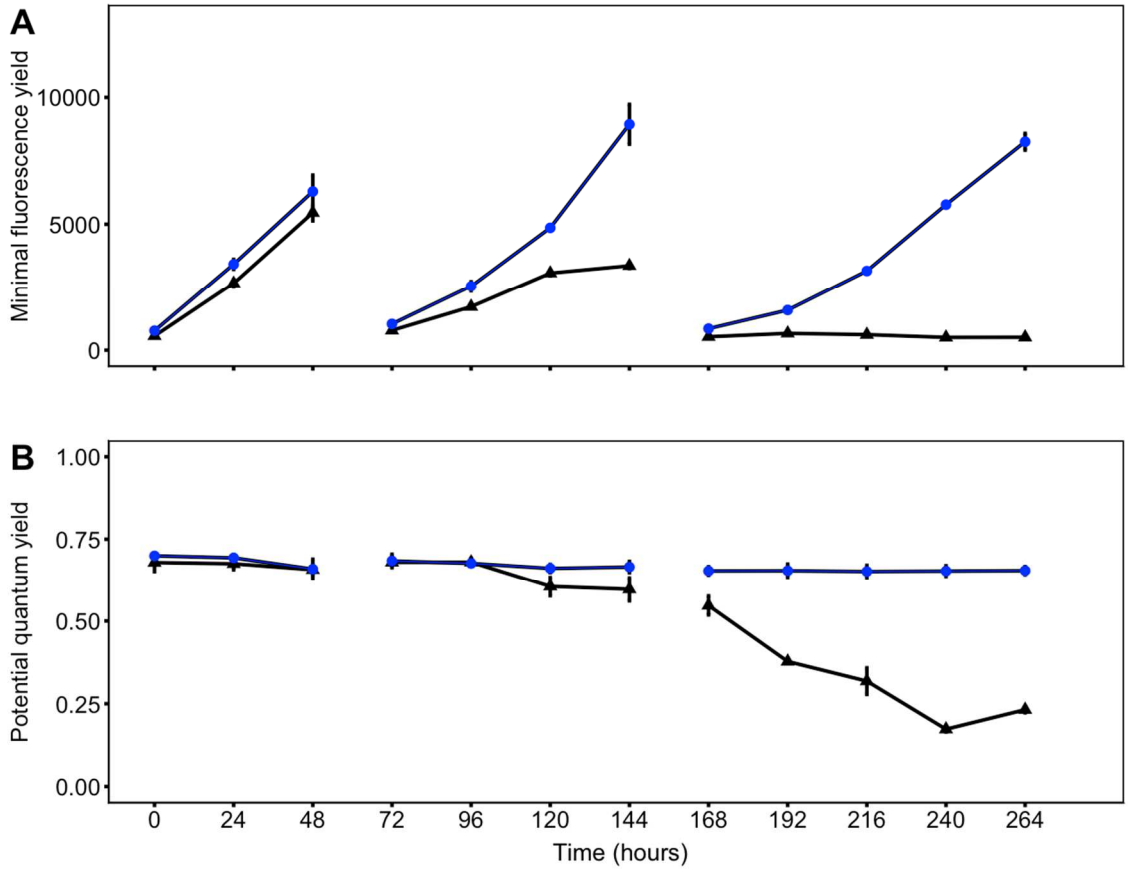



Figure 3.9. Growth of an axenic *Thalassiosira rotula* culture transferred into vitamin-free ESAW medium at 2000 cells/mL and filtrated between each growth cycle to deplete the intracellular vitamins. Blue circles depict the non-axenic *T. rotula* culture and black triangles the axenic *T. rotula* culture. After the third transfer into vitamin-free ESAW medium the axenic culture did no longer grow (A, 168 h) and its performance dropped below that of the vitamin non-axenic control (B, 168 h). Growth was monitored by minimal fluorescence ($F_0$) (A) and potential quantum yield ($F_v/F_m$) (B) with a PAM fluorometer (n = 3). Error bars depict ± 1 SD.

The non-axenic cultures of *T. weissflogii*, *T. pseudonana*, *C. closterium* and *D. brightwellii* were also depleted in repeated growth cycles of vitamins as performed for *T. rotula* above. The exhaustion of the B-vitamins decreased the growth of all four diatom species during the three consecutively growth cycles of 96 h in vitamin-free ESAW medium (Figure 3.10). However, all four diatom species started to grow in vitamin-free medium within two weeks and showed a similar growth pattern compared to the growth pattern in vitamin-containing medium (results not shown).

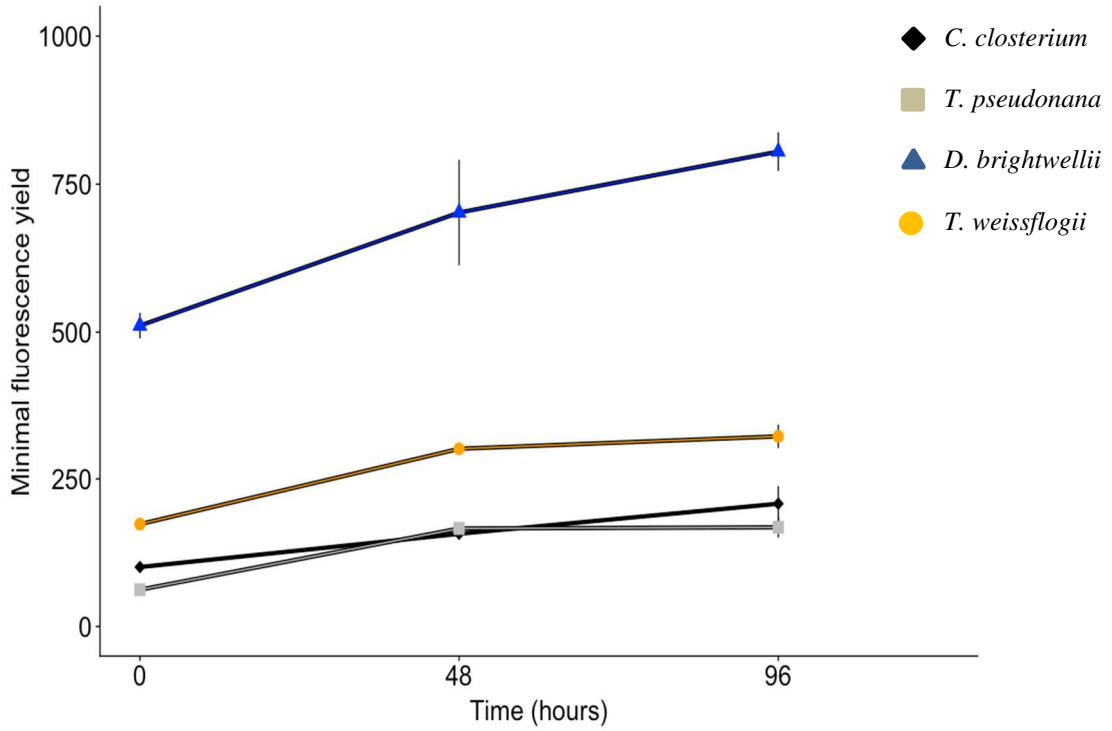



Figure 3.10. Growth of non-axenic cultures of *Cylindrotheca closterium*, *Ditylum brightwellii*, *Thalassiosira pseudonana* and *Thalassiosira weissflogii* after three consecutive filtration steps. Non-axenic cultures were 0.6 or 3 µm filtrated and cultured for 96 h. Subsequently, this filtration procedure was repeated twice. The growth of the diatoms *C. closterium*, *D. brightwellii*, *T. pseudonana* and *T. weissflogii* was flattened after the three consecutive growth cycles in vitamin-free ESAW medium. Growth was monitored by minimal fluorescence ($F_0$) (n = 3). Error bars depict ± 1 SD.

### 3.3.6 Effects of bacterial metabolites on diatom growth

The potential impact of IAA on the growth of the diatom *T. rotula* was examined. The growth of non-axenic and axenic *T. rotula* cultures was not significantly affected by the addition of IAA at concentrations of 0.1, 1, 10 and 100 nM after 240 h (Dunn's test: $p > 0.05$) (Figure 3.11). Therefore, IAA did not affect the growth of axenic and non-axenic *T. rotula* cultures.

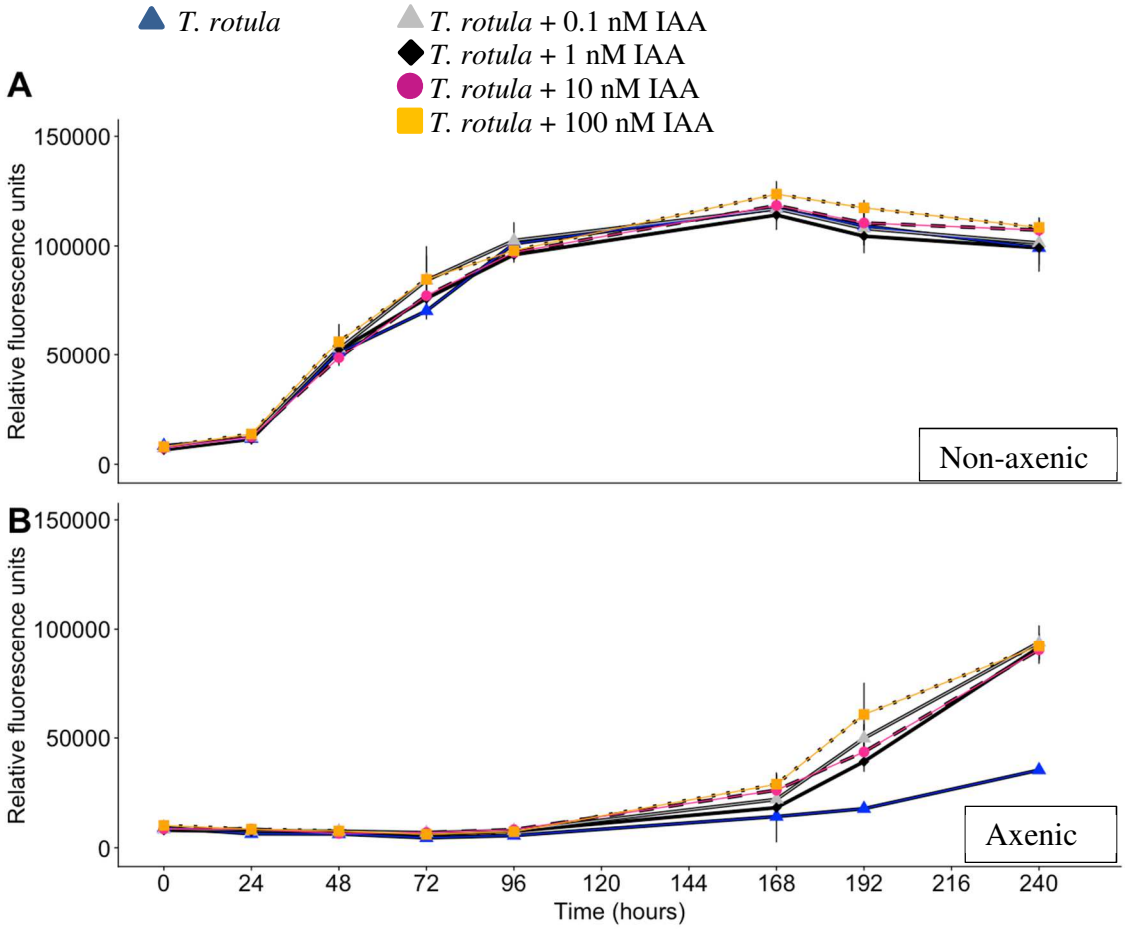



Figure 3.11 Growth of non-axenic and axenic *Thalassiosira rotula* cultures with different added concentrations of indole-3-acetic acid (IAA). IAA in the concentrations 0.1, 1, 10 and 100 nM was added to non-axenic and axenic cultures of *T. rotula*. All cultures were grown at regular growth conditions in 20 mL culture flasks (n = 3). Growth of an axenic and non-axenic *T. rotula* was not significantly affected by adding IAA in different concentrations after 240 h. Growth was monitored by relative fluorescence (RFU). Error bars depict ± 1 SD.

The potential effect of microalgal and bacterial exudates on the growth of the diatom *T. rotula* was investigated. The growth of the axenic *T. rotula* culture was not significantly affected by the inoculation of the exudates after 168 h (Tukey HSD: $p = 0.2639$) (Figure 3.12). However, the growth of the axenic *T. rotula* culture was significantly affected by the inoculation of the bacterial source community taken from *T. rotula* after 168 h (Tukey HSD: $p = 0.001$) (Figure 3.12). Therefore, the growth of axenic *T. rotula* cultures was only restored by the inoculation of bacteria (3 µm filtrate), but not by the addition of exudates (0.2µm filtrate) although both inocula were obtained from the same culture.

The hypothesis was that bacteria interacted with diatoms in the phycosphere to exchange nutrients. As the growth of the axenic *T. rotula* culture was only restored with the inoculation of bacteria but not with bacterial exudates (Figure 3.12), the fifth hypothesis was accepted.

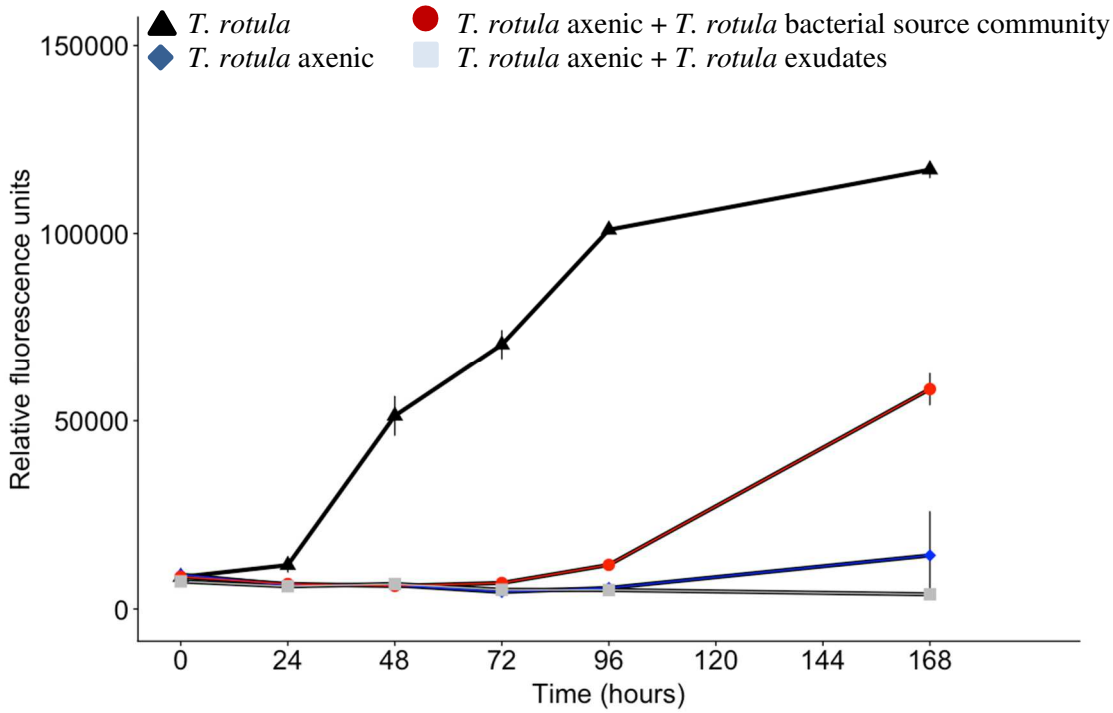



Figure 3.12. Growth of axenic *Thalassiosira rotula* co-cultured with the original bacterial source community or the exudates of the bacteria and microalgal host. Axenic cultures were inoculated with its own bacterial source community or the exudates (0.2 µm filtrate) in the diatom: bacteria ratio 1:100 and grown at regular growth conditions (n = 3). The growth of the axenic *T. rotula* culture was not significantly affected by the inoculation of bacterial exudates in the same ratio as the bacterial source community was inoculated after 168 h. Growth was monitored by relative fluorescence (RFU). Error bars depict ± 1 SD.

# Chapter 4

# 4 The marine diatom *Thalassiosira rotula* harbours a host-specific associated bacterial community under vitamin depletion

## 4.1 Introduction

The objective of this chapter was to investigate the establishment of associated bacterial communities on the diatom *Thalassiosira rotula* under vitamin absence.

Microalgae are of high relevance for the global carbon cycling (Falkowski and Raven, 2007) and it is well-known that they live in association with bacteria (Seymour et al., 2017). These bacteria can have an significant influence on the function of their microalgae host (e.g. by supply with B-vitamins; Cruz-Lopez and Maske, 2016). However, how microalgae-associated bacterial communities are shaped, and which factors influence the bacterial community establishment is not well understood. Therefore, the composition of newly shaped diatom-associated bacterial communities was investigated in these experiments to identify if the assembly of associated bacterial communities is determined by host factors, is therefore host-specific, or more generalized and determined by external factors such as bacterial source communities. For this, the experiments were conducted with the diatom *T. rotula*, which belongs to one of the most abundant and diverse genera of marine planktonic diatoms (Malviya et al., 2016). In the previous chapter, *T. rotula* was shown to be essentially dependent of B-vitamins and that the bacterial community supports diatom growth with B-vitamins under vitamin-free conditions (Chapter 3). Due to the evidenced auxotrophy of *T. rotula*, the experiments were conducted under absence of vitamins to investigate whether the growth of the diatom is affected by the recently shaped different bacterial communities.

To study which factors, contribute to the establishment of bacterial communities associated with the diatom *T. rotula* under vitamin depletion, different bacterial source

communities were dissociated from several vitamin-depleted diatom donor species and inoculated to the axenic acceptor culture of the diatom *T. rotula*. The associated bacterial community composition was evaluated in both, donor and acceptor cultures, using Illumina-MiSeq sequencing targeting the 16S rRNA V4 region. Based on the species-specificity of microalgae-associated bacterial communities that have been shown in many studies (Grossart et al., 2005; Amin et al., 2012; Behringer et al., 2018; Crenn et al., 2018), it is hypothesized that all newly shaped acceptor cultures harbour similar associated bacterial communities whereas that the donor cultures harbour unique and specific associated bacterial communities.

Furthermore, the study aimed to test if: (A) the associated bacterial community composition of *T. rotula* and *D. brightwellii* cultures (origin) is changing due to long-term cultivation (donor) (Section 4.3.2), (B) the associated bacterial community of the acceptor cultures share a certain bacterial core community (Section 4.3.2), (C) the shared bacterial taxa in the associated bacterial core community of the acceptor cultures belong to bacterial groups capable of B-vitamin biosynthesis (Section 4.3.2), and (D) the amounts of B-vitamins produced by the different newly shaped associated bacterial communities can be quantified (Section 4.3.3).

## 4.2 Material and Methods

### 4.2.1 Microalgae growth and axenic culture generation

Non-axenic microalgae cultures of *T. rotula*, *T. pseudonana*, *T. weissflogii*, *D. brightwellii* and *C. closterium* were depleted of B-vitamins (Section 3.2.4). An axenic microalga culture of *T. rotula* was generated (Section 3.2.1.5) and the intracellular stored vitamins were depleted (Section 3.2.4). The non-axenic and axenic cultures were grown in vitamin-free ESAW medium under regular growth conditions (Section 2.2.2).

### 4.2.2 Microalgae-bacteria co-culture

#### 4.2.2.1 Growth of *T. rotula* with different bacterial source communities of diatom cultures

Bacterial source communities were separated from the above described vitamin-free non-axenic diatoms by 0.6 / 3 µm gravity filtration and the density of DAPI-stained bacteria was counted under the fluorescence microscope (Section 3.2.2). Each filter was stored in 1 mL SL1 lysis buffer (NucleoSpin® Soil, MACHEREY-NAGEL) at -20°C until the donor samples were processed for Illumina amplicon sequencing. The bacterial source communities from the diatom filtrate were used to inoculate the freshly prepared axenic and vitamin-free diatom cultures to an initial diatom cell density of ~2000 cells/mL. Bacterial source communities were inoculated in a quantity to yield a ~1:100 cell ratio of diatoms to bacteria (Section 3.2.3). The following treatments were prepared: The axenic and vitamin-free *T. rotula* culture was inoculated with the bacterial source community of *T. pseudonana*, *T. weissflogii*, *D. brightwellii*, *C. closterium* and *T. rotula*, respectively. Additionally, an axenic and non-axenic *T. rotula* culture was used as control. The co-cultures were grown for 96 h (4 d) in 12-well plates (n = 3) (Section 2.2.5) under regular growth conditions (Section 2.2.2). Samples were taken every 24 h and diatom growth was monitored by the measurement of the minimal fluorescence ($F_0$) (Section 2.2.4.1). At the last timepoint (96 h), three wells (15 mL) of each treatment were 3 µm filtrated on a polycarbonate filter membrane. Subsequently, the filter was stored in 1 mL SL1 lysis buffer (NucleoSpin® Soil, MACHEREY-NAGEL) at -20°C until the acceptor samples were processed for Illumina amplicon sequencing.

#### 4.2.2.2 Identification and quantification of the essential B-vitamins

The axenic and vitamin-free *T. rotula* acceptor culture was inoculated with the bacterial source community of *T. rotula* as described in section 4.2.2.1. A sterile vitamin $B_{12}$ solution was prepared and added in the quantities of 1 and 10 pM vitamin $B_{12}$ to an axenic *T. rotula* culture of 2000 cells/mL. Additionally, an axenic and non-axenic *T. rotula* cultures were prepared in the same cell density as the control treatment. The cultures were grown in 1 L glass bottles with a culture volume of 700 mL (n = 3) (Section

2.2.5) and were rotated on a roller table with 6.2 rpm under regular light conditions with an ambient temperature of 18°C (Section 2.2.5). Samples were taken every 24 h over a period of 120 h (5 d). The growth of the diatoms was monitored by microscopic cell counting (Section 2.2.4). At the last timepoint 500 mL were taken of each culture for the vitamin quantification (Section 4.2.5).

### 4.2.3 Total bacterial DNA extraction and 16S rRNA sequencing

Samples were taken from five different donor and acceptor microalgal cultures as well as 2 freshly isolated microalgal origin cultures of *T. rotula* and *D. brightwellii* (< 1 month after isolation). Additionally, the axenic acceptor culture was used as sterile control. At the sampling timepoint, all samples except of the axenic control were in the end exponential growth phase. The alga-associated microbial community DNA was extracted from polycarbonate filters (Section 4.2.2.1) by using the "NucleoSpin® Soil" kit (MACHEREY-NAGEL). Concentration and purity of the DNA was analysed using a Nanodrop ND-1000 instrument (Thermo Fisher Scientific). Additionally, the DNA quality was verified by electrophoresis on an 1% agarose gel using DNA gel loading dye (6X) (Thermo Fisher Scientific) and GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific).

For the phylogenetic characterization of the microalgae associated microbial community, genes were amplified using an amplicon barcoded sequencing protocol for MiSeq platforms. The V4 hypervariable region of the bacterial genes were amplified using the modified universal bacterial primer set 515F/806R (515F: 5´-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTGCCAGCMGCCGCGGTAA- 3´ and 806R: 5´-GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC AGGGACTACHVGGGTWTCTAAT- 3´ Each forward and reverse primer contains different barcode sequences) with Illumina adaptor overhang sequences as previously published (Caporaso et al., 2012; Klindworth et al., 2013). The library preparation was conducted as described in the 16S metagenomic sequencing library preparation script

(https://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html, last time opened 15.07.2019, 07:12 am). The modifications from the library preparation script were as follows: The amplicon PCR was performed in triplicate for each sample to reduce the bias of the PCR. After the amplicon PCR, the replicates were pooled again for further processing. The two PCR clean-ups steps were placed on the magnetic stand until the supernatant had cleared (> 2 min).

### 4.2.4 Sequence data processing and bacterial community analysis

The amplified genes were sequenced and analysed on a MiSeq system (Illumina) following the manufacturer's instructions. Sequencing was performed in 2x 300 bps paired-end-mode using the MiSeq Reagent Kit v3. For the analysis, the trimmomatic package (Bolger et al., 2014) was used to crop the 300 bps to 275 bps and a sliding window of length 3 which allowed an average Phred quality core of 8 to filter from 5' – 3' and cut when quality dropped below 8. The paired-ends were merged with Vsearch (Rognes et al., 2016) with a minimum overlap of 40 bps and a maximum number of four mismatches. Sequences were reverse complemented, and both directions merged into one file. The combined files were then filtered (allowing 10% mismatch and a minimum overlap of 17 bps for forward and 13 bps for reverse) for the existence of the primer sequences (forward -> reverse complemented) and primer sequences were removed. This filtration step was followed by feature filtering which allowed a maximum expected error per sequence of 1, minimum length of 275 bps, maximum length of 475 bps and maximum number of ambiguities of 0. In the same step the headers were renamed by a shal digest of the sequence itself. Each sample was dereplicated independently (abundance of each amplicon added to the header) and chimera checked *de novo*. All samples were pooled and dereplicated in total to produce a combined dataset. The combined dataset served as input for the swarm operational taxonomic unit (OTU) clustering with a distance of 1 (Mahe et al., 2014). The most abundant amplicon of an OTU cluster was used as representative.

The swarm output was run on the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3) (Quast et al., 2013). Every read was aligned operating the SILVA Incremental Aligner (SINA v1.2.10 for ARB SVN (revision 21008)) (Pruesse et al., 2012) against the SILVA SSU rRNA SEED and quality controlled (Quast et al., 2013). Reads shorter than 50 aligned nucleotides and exceeding 2% of ambiguities or homopolymers, respectively, were excluded from further processing. Putative contaminations and artefacts read with a low alignment quality (50 alignment identity, 40 alignment score reported by SINA), were identified and excluded from downstream processing.

Following the initial quality control steps, identical reads were identified (dereplication) on a per sample basis and the reference read of each OTU was classified. Dereplication was done using cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit, last time opened 15.07.2019, 07:19 am) (Li and Godzik, 2006) operating in *accurate mode*, ignoring overhangs, and applying identity criteria of 1.00 and 0.1, respectively. The classification was performed by local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 132; http://www.arb-silva.de, last time opened 15.07.2019, 07:20 am) using blastn (version 2.2.30+; http://blast.ncbi.nlm.nih.gov/Blast.cgi, last time opened 15.07.2019, 07:21 am) with standard settings (Camacho et al., 2009).

The classification of each OTU reference read was mapped onto all reads that were assigned to the respective OTU. Delivering quantitative information (number of individual reads per taxonomic path), within the limitations of PCR and sequencing techniques biases, as well as multiple rRNA operons. Reads without any BLAST hits or reads with weak BLAST hits, where the function "(% sequence identity + % alignment coverage) / 2" did not exceed the value of 93, remained unclassified. These reads were assigned to the meta group "No Relative" in the SILVAngs fingerprint and Krona charts (Ondov et al., 2011).

Bacterial community patterns were examined for differences among donor and acceptor cultures and within donor and acceptor cultures and of short-term (< 1 month)

and long-term (> 1 year) cultivation in the laboratory. Non-parametric (multivariate) analyses were undertaken using the standardized abundance data normalized to the median sequencing depth. Multidimensional scaling was applied to provide an ordination of the data using Bray-Curtis dissimilarities. Principal coordination analysis (PCoA) was then used to investigate differences between the diverse cultures. To identify the OTUs which were present across the acceptor cultures, a Venn diagram was generated (http://bioinformatics.psb.ugent.be/webtools/Venn/, last time opened 15.07.2019, 07:11 pm). The community structure was analysed in R (R Core Team, 2018) with Phyloseq (McMurdie and Holmes, 2013), vegan (Oksanen et al., 2018) and plotted with ggplot2 (Wickham, 2009).

### 4.2.5 Determination of B-vitamin concentrations in microalgae cultures

#### 4.2.5.1 Preconcentration of samples

500 mL of each diatom culture (Section 4.2.2.2) were immediately vacuum filtered over a 0.2 µm pore-size polycarbonate membrane filter (Nucleopore Track-Etched Membranes, Whatman®) and the filtrate was stored in the dark at 4°C until further processing. The preconcentration took place within 24 h after the filtration using a modification of a previously published method (Heal et al., 2014). A glass chromatography column (Diameter: 20, Length: 400 mm) was packed with 10 g Diaion® HP-20 (Sigma-Aldrich). The HP-20 was conditioned for 10 min in 60 mL of HPLC grade (VWR) methanol and subsequently washed with at least 60 mL of Milli-Q water. The 500 mL samples were adjusted to pH 5.5 - 6.5 with formic acid and gravity loaded onto the column at 7 mL/min. The column was then washed with 60 mL Milli-Q adjusted to a pH of 6.0 - 6.5. Samples were eluted from the column with 60 mL methanol and dried in the speed vac (Savant, Thermo Fisher Scientific). Finally, samples were reconstituted in 500 µL of Milli-Q water (95%) and LC-MS grade methanol (5%). To minimize photodegradation of the vitamins, the filtration and preconcentration was performed in a shaded room and bottles and vials were covered with aluminium foil.

#### 4.2.5.2 Recovery of vitamins during solid phase extraction

To determine the percentage recovery of cyanocobalamin (vitamin $B_{12}$) (Table 4.1) at different concentrations during solid phase extraction (SPE), the 0.2 µm filtrated ESAW medium was spiked with known amounts of vitamin $B_{12}$ before preconcentration on SPE. All samples were analysed in the same way. These values of recovery were calculated to correct the vitamin concentrations in microalgae-bacteria co-culture experiments in ESAW medium.

Table 4.1. Results of recovery analysis (with standard deviation of triplicate analysis) of different cyanocobalamin (vitamin $B_{12}$) concentrations in ESAW medium.

| Analyte | Concentration (pM) | % Recovery |
|---|---|---|
| Cyanocobalamin | 1 | 37 ± 21 |
| | 10 | 86 ± 29 |
| | 100 | 82 ± 4 |
| | 10000 | 83 ± 3 |

#### 4.2.5.3 UPLC/MS conditions

The UPLC/electrospray ionization (ESI)-MS method was adapted from a previously published method (Heal et al., 2014). All samples and standards were analysed on a LC-MS / MS system (UPLC: I-Class, MS /MS: Xevo; Waters). A UPLC BEH C18 reverse phase column (Acquity 50 mm x 2.1 mm, 1.7 µm, Waters) was used with a flow-rate of 0.5 mL/min for the first 5 min and 0.6 mL/min for the last 1.5 min at 40°C. The gradient elution was performed with the two eluents water (eluent A) and methanol (eluent B), both containing 10 mM ammonium formate and 0.1% formic acid. Initial conditions were 2 min column equilibration with 99% A and 1% B, followed by a linear gradient to 45% A and 55% B in 2 min, followed by a linear gradient to 100% B in 0.5 min, and isocratic elution for 0.5 min with 100% B, followed by linear gradient to 99% A and 1% B in 0.2 min, and isocratic elution for 1.3 min at 99% A and 1% B. All analyses were performed in positive ion mode using multiple reaction monitoring (MRM). The ESI source temperature was 55°C with a desolvation temperature of 600°C.

Collision energies, cone voltages, and retention times of cobalamin are listed in Table 4.2. Peak areas of the listed transitions (see Table 4.2) were used for identification and quantification.

Table 4.2. Conditions of mass spectrometry (MS) and retention times (RT) for each analyte. Multiple reaction monitoring (MRM) was used to identify and quantify each precursor and product pair at the indicated collision energy (CE) and cone voltage (CV). The listed product ion was used to quantify the analyte and the second listed product (second row of the same analyte) used to confirm the identity of the analyte.

| Analyte | MRM | CE (V) | CV (V) | RT (min) |
|---|---|---|---|---|
| Hydroxocobalamin | 676.25 -> 147.10 | 36 | 30 | 0.49 - 5.00 |
| Adenosylcobalamin | 790.10 -> 147.10 | 36 | 30 | 0.49 - 5.00 |
| Adenosylcobalamin | 790.10 -> 665.60 | 36 | 30 | 0.49 - 5.00 |
| Methylcobalamin | 673.10 -> 147.10 | 36 | 30 | 0.49 - 5.00 |
| Methylcobalamin | 673.10 -> 685.60 | 36 | 30 | 0.49 - 5.00 |
| Cyanocobalamin | 678.25 -> 147.10 | 36 | 30 | 0.49 - 5.00 |
| Cyanocobalamin | 678.25 -> 359.20 | 24 | 30 | 0.49 - 5.00 |
| Cyanocobalamin | 678.25 -> 912.30 | 36 | 30 | 0.49 - 5.00 |
| Cyanocobalamin | 678.25 -> 997.35 | 24 | 30 | 0.49 - 5.00 |

### 4.2.6 Statistical analysis

Differences in algal abundance at the last measurement timepoint between the axenic acceptor culture, the various acceptor cultures inoculated with different bacterial source communities and the different vitamin $B_{12}$ levels (Section 4.3.1 & 4.3.2) were analysed by One-way Analyses of Variance (ANOVA) followed by Tukey HSD (normally distributed data). Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's-test). All statistical tests were performed at a significance level of 0.05. The analyses and plots were done using R (R Core Team, 2018) with the packages dplyr (Hadley et al., 2018), ggpubr (Kassambara, 2018), cowplot (Wilke, 2017), car (Fox and Weisberg, 2011) and ggplot2 (Wickham, 2009).

## 4.3 Results and Discussion

### 4.3.1 Microalgae-bacteria co-culture

The *T. rotula* acceptor culture inoculated with its own bacterial source community grew less than the non-axenic vitamin deplete control after 96 h (Tukey HSD, $p < 0.001$) (Figure 4.1). In contrast, the axenic acceptor culture revealed stagnant growth during the entire experiment duration. The axenic *T. rotula* acceptor cultures inoculated with the bacterial source communities obtained from different diatom species reached higher $F_0$ values than the *T. rotula* acceptor culture inoculated with the original *T. rotula* bacterial source community after 96 h (Figure 4.1, Table 4.3). This ubiquitous growth restoration of the vitamin-free axenic *T. rotula* culture by the inoculation of different bacterial source communities suggested that all five newly established bacterial communities on the acceptor cultures harboured bacteria capable of B-vitamin biosynthesis (Figure 4.1). Cruz-Lopez and Maske (2016) showed that the bacterial community obtained from natural seawater supplied an axenic and vitamin-depleted dinoflagellate culture with vitamin $B_1$ and $B_{12}$. Thus, the demand for B-vitamins of diverse microalgal species can be fulfilled by different microalgal microbiomes and bacterial communities from natural seawater.

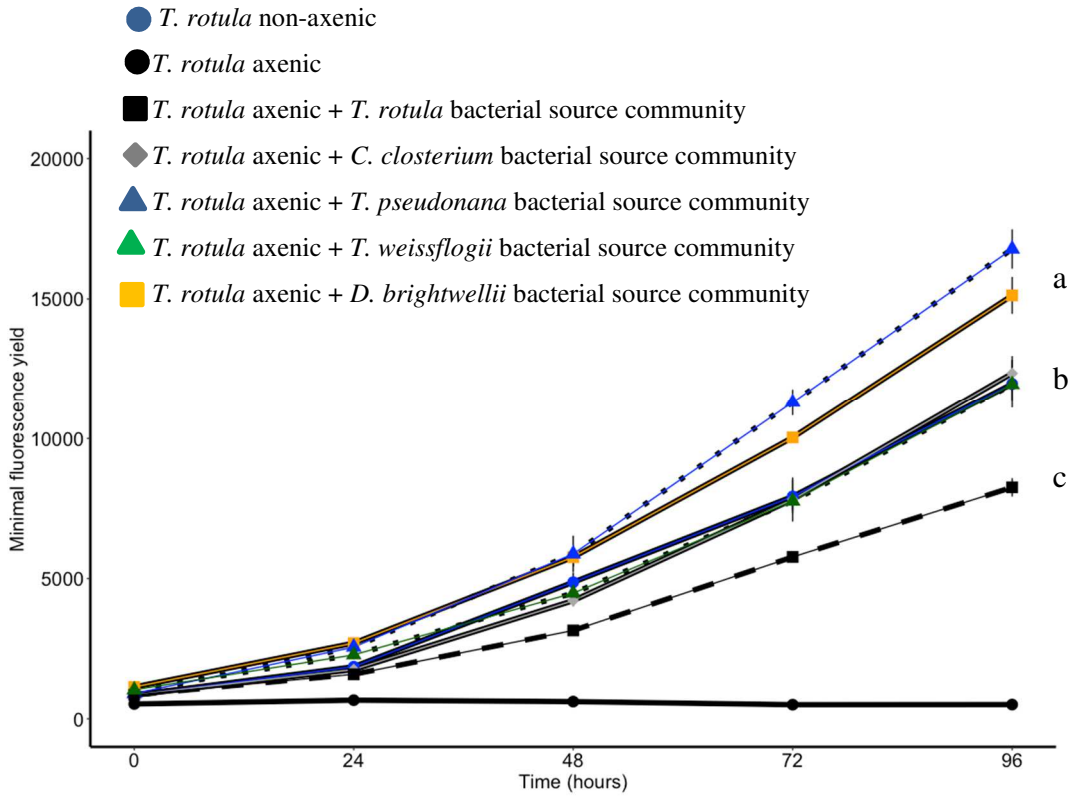



Figure 4.1. Growth of axenic *Thalassiosira rotula* acceptor cultures under absence of vitamins in co-culture with different inoculated bacterial source communities obtained from *T. rotula*, *Ditylum brightwellii*, *Cylindrotheca closterium*, *Thalassiosira pseudonana* and *Thalassiosira weissflogii*. Algal growth was determined by minimal chlorophyll fluorescence ($F_0$) (n = 3). The letters indicate significant differences between treatments assigned with another letter: Tukey HSD $p < 0.01$. Error bars depict ± 1 SD.

Table 4.3. Pairwise comparison of growth of *Thalassiosira rotula* acceptor cultures after 96 h (4 d) with the original versus bacterial source communities obtained from different diatom species (Tukey HSD).

| Pairwise comparison after 96 h of growth of *T. rotula* acceptor cultures inoculated with bacterial source communities of: | *p*-value (Tukey HSD) |
|---|---|
| *T. rotula* vs *Cylindrotheca closterium* | < 0.001 |
| *T. rotula* vs *Thalassiosira weissflogii* | < 0.001 |
| *T. rotula* vs *Thalassiosira pseudonana* | < 0.001 |
| *T. rotula* vs *Ditylum brightwellii* | < 0.001 |

To investigate the vitamin B requirements of the axenic *T. rotula* acceptor culture, the above-described experiment was repeated to identify and quantify the essential B-vitamins for the diatom growth. The axenic acceptor culture inoculated with the original *T. rotula* bacterial source community and the vitamin-free non-axenic control grew significantly better than the axenic vitamin-free control after 120 h (Figure 4.2; Tukey HSD, $p < 0.02$), confirming the above described vitamin supply by the *T. rotula* bacterial community. Similarly, the axenic acceptor culture inoculated with 1 pM and 10 pM vitamin $B_{12}$ grew significantly better than the axenic vitamin-free control after 120 h (Figure 4.2; Tukey HSD, $p < 0.02$) and the growth was equal to the non-axenic control after 120 h (Figure 4.2; Tukey HSD, $p = 1$). However, the growth of the axenic *T. rotula* was the same between both added vitamin $B_{12}$ quantities after 120 h (Figure 4.2; Tukey HSD, $p = 1$). The experiment demonstrated that the B-vitamin auxotroph diatom *T. rotula* (Chapter 3) requires only 1 pM vitamin $B_{12}$ for growth (Figure 4.2). Thus, the growth of the diatom *T. rotula* is essentially dependent on extracellular vitamin $B_{12}$, but not on extracellular vitamin $B_1$ and $B_7$ (Figure 4.2). The tested quantities of vitamin $B_{12}$ did not significantly affect the growth of *T. rotula*, but both quantities restored the growth of the axenic and vitamin-free acceptor culture of *T. rotula*. The raise of the vitamin $B_{12}$ concentration in the medium did not further enhanced algal growth is a contrary result to the outcomes of another study that showed that the abundance of the green alga *Lobomonas rostrata* increased due to enhanced concentrations of vitamin $B_{12}$ (Kazamia et al., 2012).

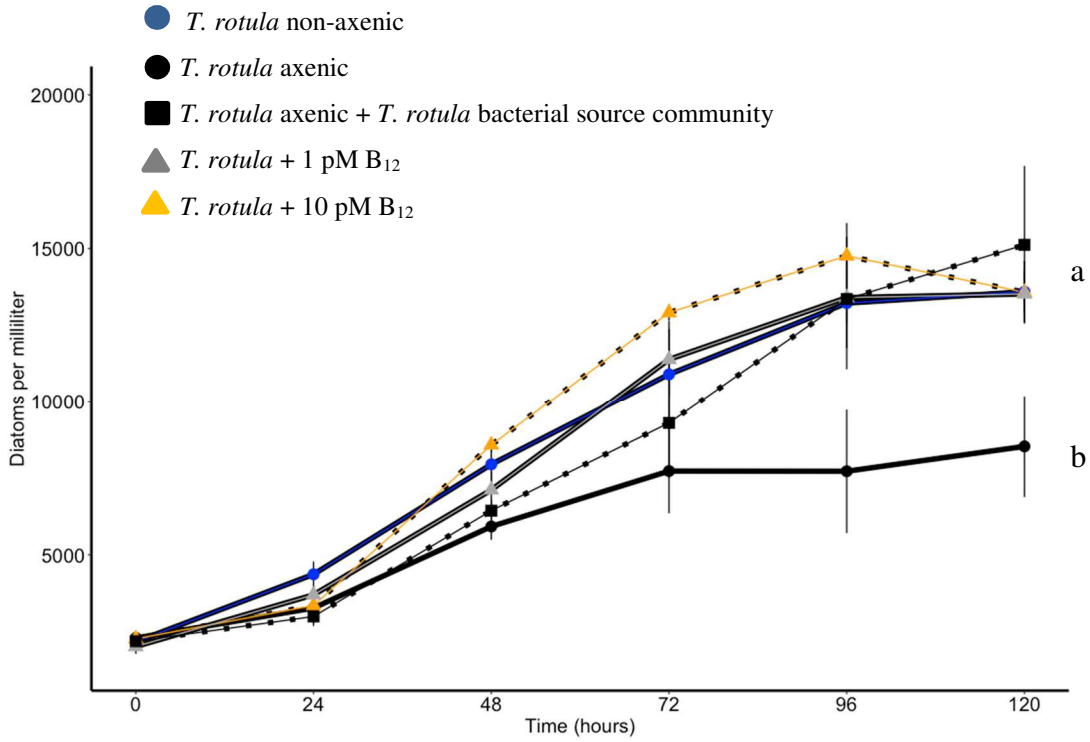



Figure 4.2. Growth of axenic *Thalassiosira rotula* acceptor cultures under absence of vitamins in co-culture inoculated with the *T. rotula* bacterial source community and with 1 pM and 10 pM vitamin $B_{12}$. Algal abundance was determined by microscopic cell counts (n = 3). The letters indicate significant differences between treatments assigned with another letter: Tukey HSD $p < 0.02$. Error bars depict ± 1 SD.

### 4.3.2 Community composition of microalgae-associated bacterial communities

In this chapter, it was shown that the growth of the axenic and vitamin-free acceptor culture could be restored by the inoculation of bacterial source communities obtained from different donor diatom cultures. To analyse the associated microbial communities of the donor and acceptor cultures as well as of the two freshly isolated microalgal strains, 16S rRNA gene amplicon sequencing was performed. The clustering of bacterial 16S rRNA gene amplicons was done at a 98% similarity level. Overall, 462090 raw reads were generated in this study, whereof 162489 reads were affiliated to the domain of bacteria. The following analysis resulted in the identification of 27 - 117 OTUs per acceptor, donor or origin culture (Figure 4.3) and a total of 372 OTUs were identified. Rarefaction analysis showed a variable sequencing depth (Figure 4.3). Therefore, all samples were normalized to the median sequencing depth to reduce the

variability between samples caused by the bias of NGS sequencing. The axenic control of *T. rotula* shows a flatter curve in the rarefaction curve compared to all other treatments (Figure 4.3, black dotted line) as only chloroplast and mitochondrial sequences and some negligible bacterial sequences were read during sequencing. However, no bacteria were detectable by epifluorescence microscopy after DAPI staining before and after the experiment, indicating sterile conditions throughout the whole experiment.

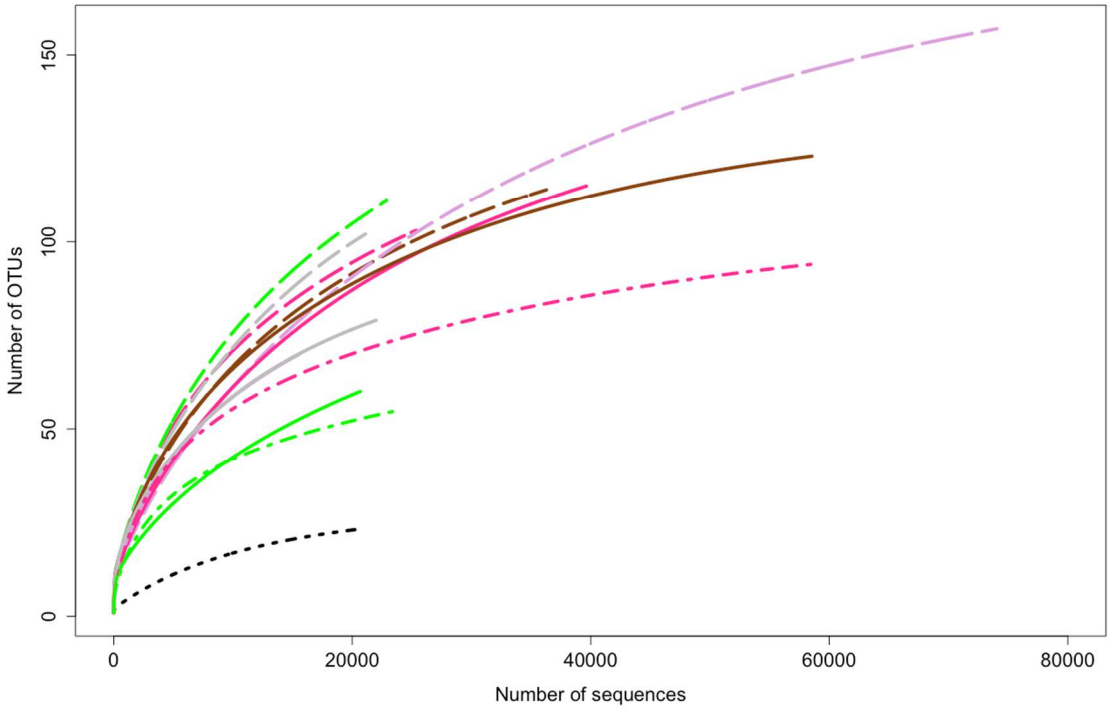



Figure 4.3. Rarefaction curves for each of the sequenced bacterial community samples (with OTUs assigned at a 98% similarity cut-off). The grouping into donor, acceptor, origin, control samples is depicted by the different line types: solid: donor; long dash: acceptor; dot dash: origin; dotted: control.

The phylogenetic analysis of bacteria attached to the donor and origin cultures of *T. rotula* and *D. brightwellii* revealed variations in the associated bacterial community composition among short-term (< 1 month) and long-term cultivation (> 1 year) (Figure 4.4). Interestingly, the diversity of bacterial taxa increased due to longer cultivation under laboratory conditions in both microalgal species (Figure 4.4). Although both microalgal species were isolated at the same sampling location and sampling season, the associated

bacterial community composition in the origin algal cultures were distinct from each other.

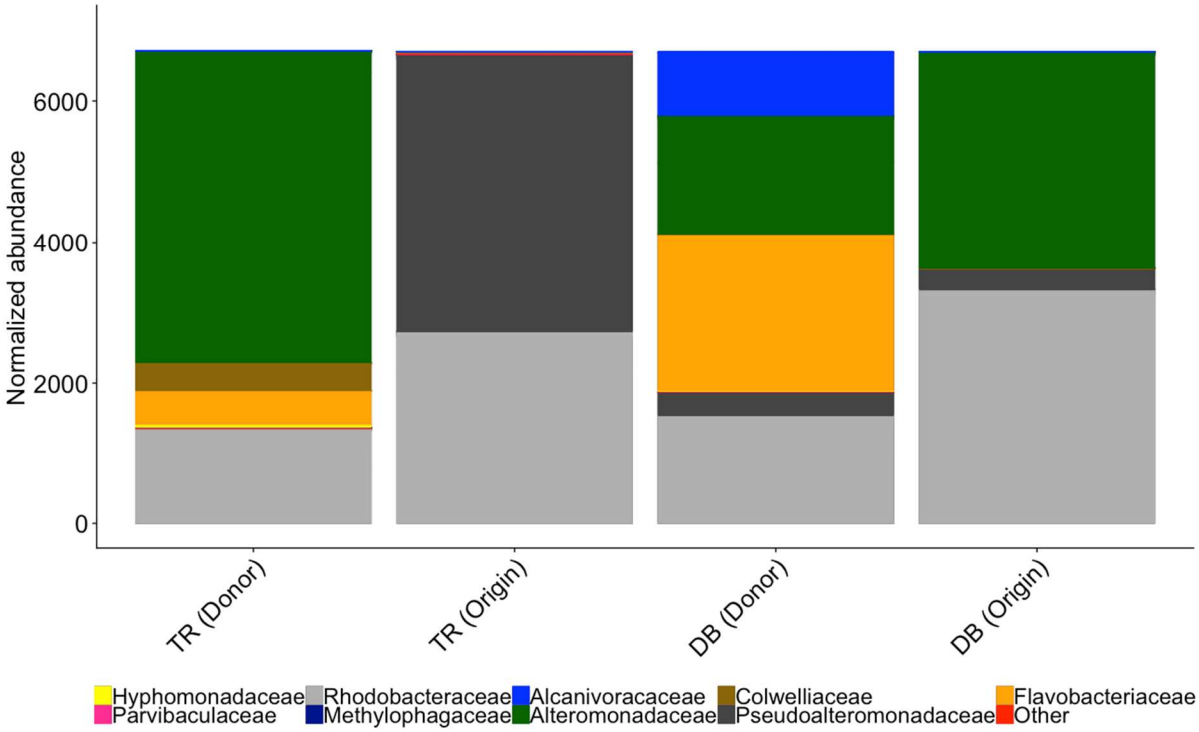



Figure 4.4. Phylogenetic composition of associated bacterial communities on the family level attached to *Thalassiosira rotula* (TR) and *Ditylum brightwellii* (DB). The term in brackets describes the source of the samples: donor: > 1 year of lab cultivation, origin: < 1 month of lab cultivation. The normalized abundance of OTUs was analysed with R using the Phyloseq package.

The comparison of the associated bacterial community composition of the donor algal cultures revealed that all donor communities were different from each other at the resolution of family and genus (Figure 4.5 & 4.6). The only exceptions were the donor cultures of *T. pseudonana* and *T. weissflogii* which were similar to each other except for the presence of *Parvibaculaceae* in the donor culture of *T. weissflogii* (Figure 4.5).

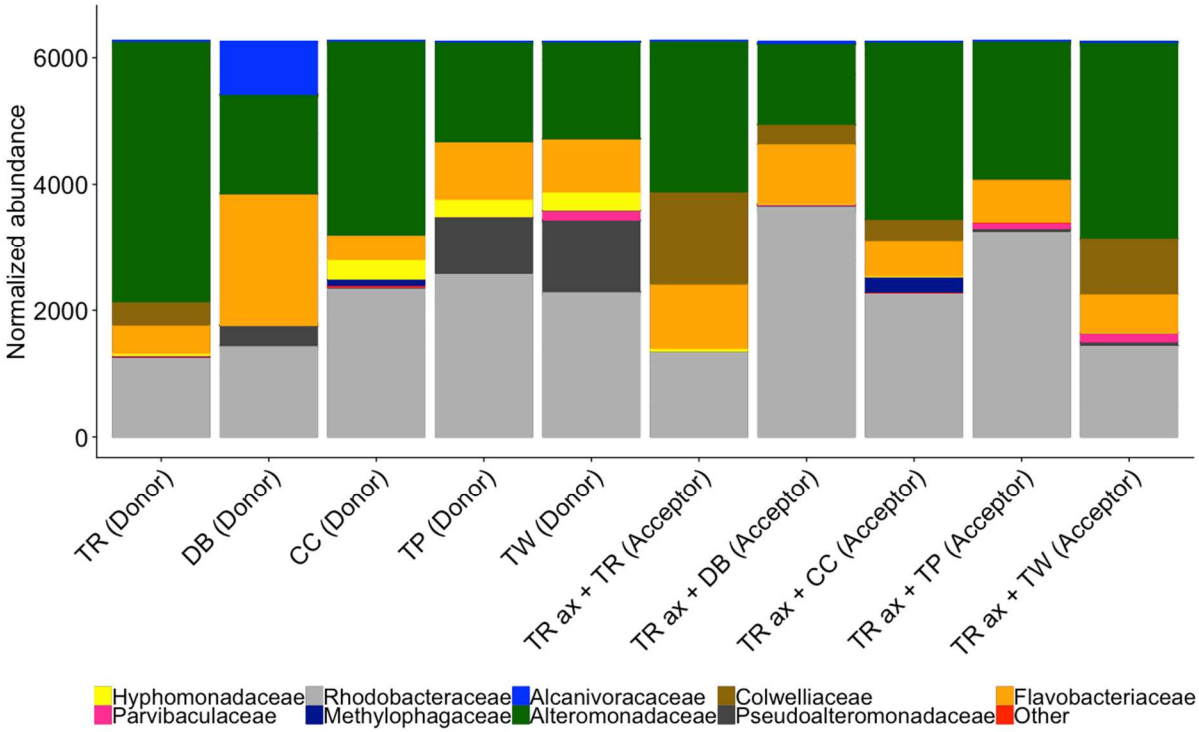



Figure 4.5. Phylogenetic composition of associated bacterial communities on the family level attached to *Thalassiosira rotula* (TR), *Ditylum brightwellii* (DB), *Cylindrotheca closterium* (CC), *Thalassiosira pseudonana* (TP), and *Thalassiosira weissflogii* (TW). The term in brackets describes if this is the bacterial source community (donor) or the newly established associated bacterial community on *T. rotula* (acceptor). The normalized abundance of OTUs was analysed with R using the Phyloseq package.

The genera *Alteromonas* and *Sulfitobacter* constituted more than 50% of all OTUs in the associated bacterial communities of the acceptor cultures, while these two genera were less abundant in most of the associated bacterial communities in the donor cultures (Figure 4.6). Thus, the acceptor cultures harboured mainly Gammaproteobacteria and Alphaproteobacteria in their associated bacterial community, which are described to be important vitamin $B_{12}$ producers in the ocean (Sanudo-Wilhelmy et al., 2014; Doxey et al., 2015). Within the Alphaproteobacteria class, the most commonly abundant order in the acceptor and donor associated bacterial communities were the Rhodobacterales. The order of Rhodobacterales has been identified as a potential algal symbiont that provides microalgae with vitamin $B_{12}$ in exchange for DOC (Wagner-Dobler et al., 2010). It is remarkable that 86% of the Rhodobacterales possess the *de novo* pathway for vitamin $B_{12}$ synthesis (Sanudo-Wilhelmy et al., 2014), which might explain the high percentage of these bacteria in the vitamin depleted donor and acceptor cultures. The potential

significance of the Rhodobacterales to the donor and acceptor algae can be supported by the absence of chemoautotrophic Thaumarchaeota and photoautotrophic cyanobacteria in all analysed algal cultures. Both prokaryotes are known as important vitamin B producers in the ocean besides selected Proteobacteria (Heal et al., 2017).

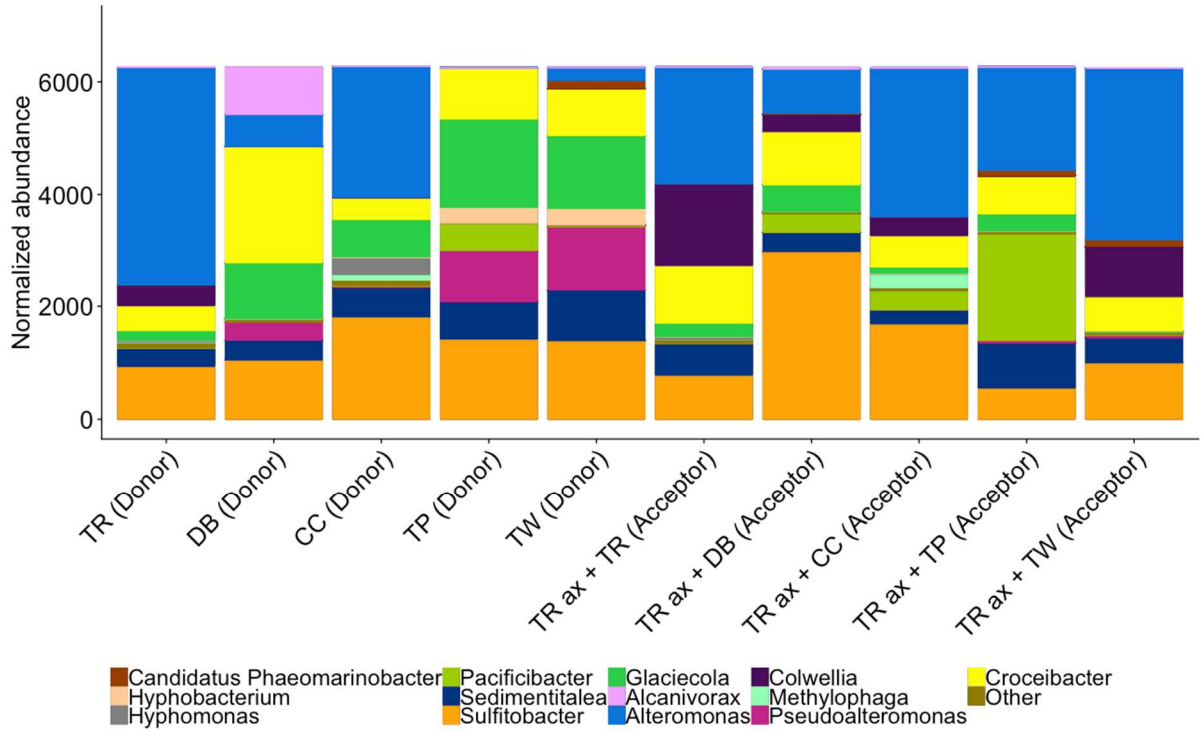



Figure 4.6. Phylogenetic composition of associated bacterial communities on the genus level attached to *Thalassiosira rotula* (TR), *Ditylum brightwellii* (DB), *Cylindrotheca closterium* (CC), *Thalassiosira pseudonana* (TP), and *Thalassiosira weissflogii* (TW). The term in brackets describes if this is the bacterial source community (donor) or the newly established associated bacterial community on *T. rotula* (acceptor). The normalized abundance of OTUs was analysed with R using the Phyloseq package.

In this chapter, the question was addressed if microalgal species influenced the bacterial composition of the associated bacterial communities and if long-term cultivation resulted in changes in the microbiome. The PCoA, based on Bray-Curtis dissimilarities matrix, showed that the bacterial communities associated with *D. brightwellii* and *T. rotula* in the origin and donor culture differed between both diatom species (Figure 4.7A). The PCoA also slightly separated the donor and origin associated bacterial communities of the diatoms *T. rotula* and *D. brightwellii* (Figure 4.7A), indicating that the bacterial composition of the microbiomes did not undergo major changes due to long-

term cultivation. Thus, the observation of Behringer et al. (2018) that long-term cultivation only resulted in small changes in the associated bacterial community composition can be underpinned with two more diatom species from this thesis.

The PCoA did not separate the different acceptor associated bacterial communities except of *T. pseudonana* and *D. brightwellii* (Figure 4.7B & 4.7D). In contrast, most of the donor associated bacterial communities were separated from each other in the PCoA (Figure 4.7B & 4.7C). Only the donor bacterial communities associated with *T. pseudonana* and *T. weissflogii* clustered together in the PCoA, two diatom species which were already cultivated in the laboratory for many years (Figure 4.7C). The differences in the associated bacterial community compositions of the donor cultures indicate that the different diatoms harbour a unique and specific bacterial community. The results complemented, however, previous studies suggesting that microalgal cultures harbour specific bacterial communities (Grossart et al., 2005; Krohn-Molt et al., 2017; Behringer et al., 2018; Crenn et al., 2018). This distinction of bacterial communities associated with microalgae may be due to differences in the DOC composition produced by different microalgal species that in turn can support different groups of bacteria. Consequently, the similarity of the newly established acceptor associated bacterial communities resulted most likely from a specific DOC release of the diatom *T. rotula* (Figure 4.7C & 4.7D). Especially the similarity of the newly established acceptor associated bacterial communities with the donor associated bacterial community of the original *T. rotula* in the PCoA indicates that the similarity was caused by a specific DOC release of *T. rotula*. These results suggest that the associated bacterial community of *T. rotula* is host-specific.

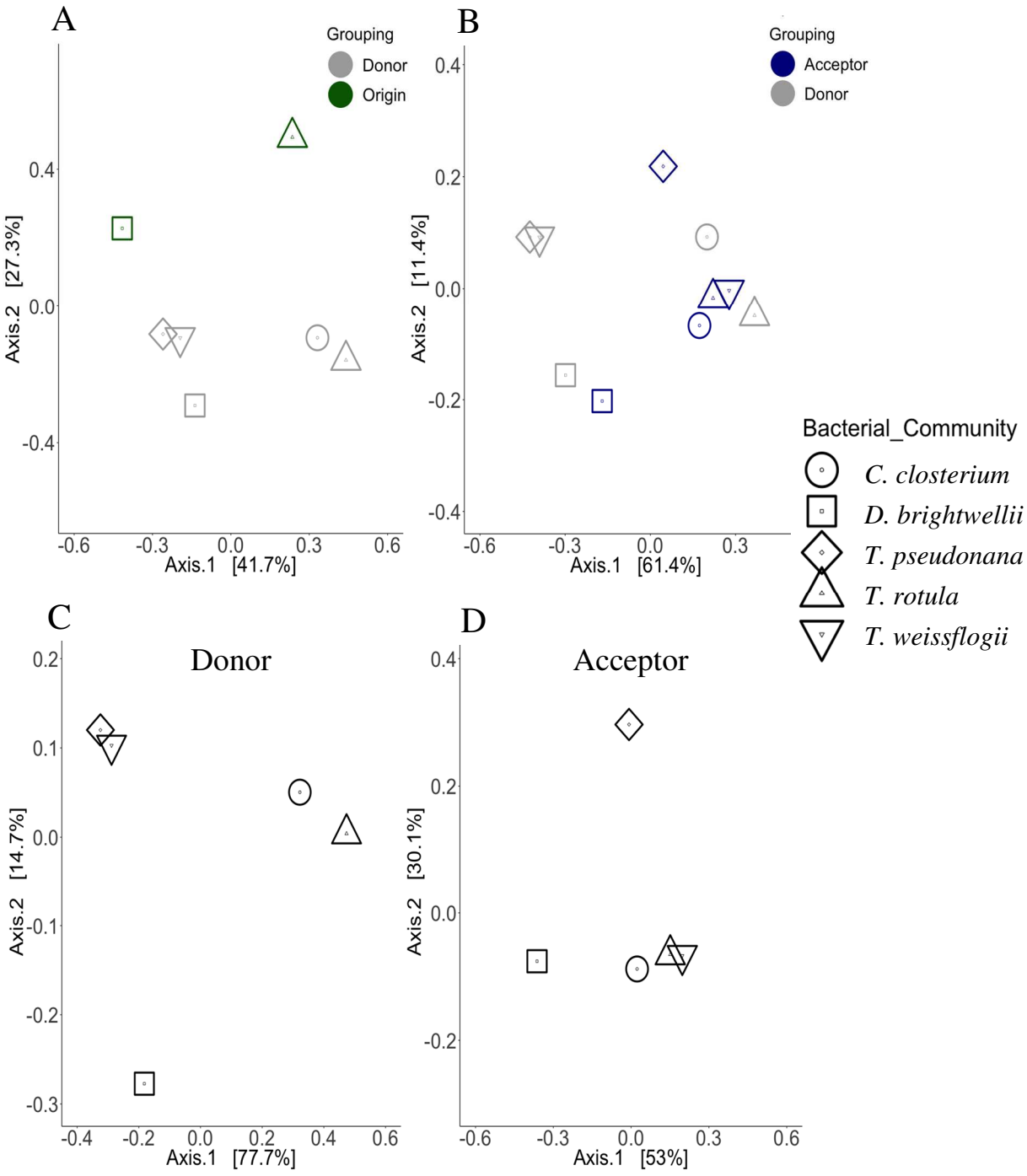



Figure 4.7. Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity. The PCoA revealed that the origin and donor associated bacterial communities were separated and that the acceptor associated bacterial communities were not separated. (A) Bray-Curtis PCoA ordination of pooled origin and donor associated bacterial communities. (B) Bray-Curtis PCoA ordination of pooled donor and acceptor associated bacterial communities. (C) Bray-Curtis PCoA ordination of donor associated bacterial communities. (D) Bray-Curtis PCoA of acceptor associated bacterial communities.

To identify the bacterial taxa that were commonly present in the acceptor associated bacterial communities, a Venn diagram was generated. The results of the Venn analysis of the acceptor cultures revealed that all five-acceptor associated bacterial communities commonly shared 17 bacterial taxa (Figure 4.8), which belong to *Rhodobacteraceae*, *Alteromonadaceae*, *Flavobacteriaceae* and *Colwelliaceae*. The investigation of the contribution of the 17 shared bacteria in the acceptor associated bacterial communities revealed that the bacteria belong exclusively to the three bacterial orders of Alteromonadales (47%), Rhodobacterales (29%) and Flavobacteriales (24%). The observed ubiquitous bacteria in the acceptor microbiomes are supported by several studies that showed that Alteromonadales, Rhodobacterales and Flavobacteriales are strongly associated with diatoms (Grossart et al., 2005; Kaczmarska et al., 2005; Sapp et al., 2007; Cruz-Lopez and Maske, 2016; Behringer et al., 2018). Consequently, *T. rotula* harbours a putative associated bacterial core community of 17 bacterial taxa of four different bacterial families.

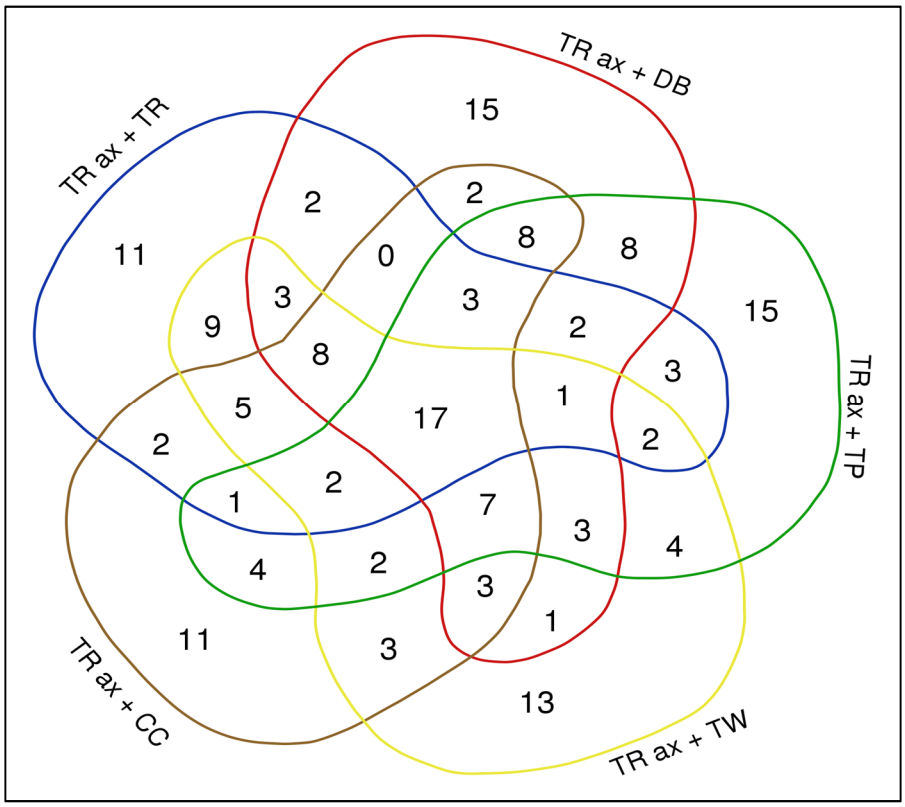



Figure 4.8. Venn diagram showing the number of bacterial taxa uniquely present in one or commonly present in two, three, four or five of the algae associated bacterial communities in the acceptor cultures. Meaning of colour is *Thalassiosira. rotula* axenic (TR ax) inoculated with the bacterial source communities: *T. rotula* (TR): blue, *Ditylum brightwellii* (DB): red, *Thalassiosira pseudonana* (TP): green, *Thalassiosira weissflogii* (TW): yellow, *Cylindrotheca closterium* (CC): brown.

### 4.3.3 Determination of B-vitamin concentrations in microalgae cultures

The crucial vitamin $B_{12}$ concentration for growth of *T. rotula* was determined to be ~ 1 pM. The adopted method achieved a detection limit of 1 pM for vitamin $B_{12}$ with a recovery rate of 37 - 86 % (Table 4.1). Although the accomplished detection limit was in the range of the required vitamin $B_{12}$ for growth of *T. rotula*, it was not possible to detect any extracellular vitamin $B_{12}$ in the acceptor cultures. Furthermore, even if 1 or 10 pM $B_{12}$ were added to the axenic acceptor culture instead of the inoculation of the bacterial source community, no extracellular vitamins were detected in the vitamin-supplemented cultures.

In the ocean, vitamin $B_{12}$ occurs in low concentrations and is only produced by selected bacteria and archaea (Croft et al., 2005; Sanudo-Wilhelmy et al., 2014). Hence, it could be the case that microalgae rapidly ingest the newly synthesized vitamins to fulfil their essential requirements as long as the vitamins are available. This response of microalgae to nutrient shortage is a well-known strategy to scope with variable nutrient regimes and is described as luxury consumption (Sommer, 1985). In chapter 3 it was shown that the axenic *T. rotula* culture was able to store enough vitamins to grow for at least 5 to 7 d without an exogenous B-vitamin source. Consequently, *T. rotula* is able to store vitamins for shorter periods when vitamins become scarce. The capability of *T. rotula* to intracellular store vitamins could be a reason why it was not possible to quantify any vitamin $B_{12}$ in the cultures. Another reason why no vitamin $B_{12}$ could be quantified in the diatom cultures might be the reduction of cyanocobalamin (vitamin $B_{12}$) to hydroxocobalamin. The cobalamins have different bio-and photochemistries, but all forms of cobalamins are light sensitive (Juzeniene and Nizauskaite, 2013). However, for the calibration of the LC-MS only a standard solution of cyanocobalamin was available. Thus, the potential degradation product of the active forms, the hydroxocobalamin, was measured, but could not be calibrated to identify and decrease the detection limit.

To prevent photodegradation of vitamins during preconcentration, all steps were conducted in the dark when possible. However, some light was necessary to be able to operate the SPE and avoid the column to fall dry during extraction. Therefore, photodegradation of vitamins during the preconcentration cannot be completely excluded. To test how quickly dissolved vitamin $B_{12}$ is photodegraded, cyanocobalamin was dissolved in ESAW medium and illuminated with ca. 30 – 70 µmol photons $m^{-2}$ $s^{-1}$ in the same culturing container in which the quantification experiment was performed. The light intensity of 30 - 70 µmol photons $m^{-2}$ $s^{-1}$ was the same as during the co-culturing experiment and clearly in excess of the light intensity that the samples were exposed to during vitamin $B_{12}$ preconcentration. The vitamin $B_{12}$ concentration was measured in triplicate before and after fully lighted for 5 d and resulted to be similar in both samples from before and after illumination (results not shown). Consequently, photodegradation of vitamin $B_{12}$ was unlikely during the co-culturing experiment and the preconcentration, because the vitamin concentration did not change during illumination with

ca. 30 - 70 µmol photons $m^{-2}$ $s^{-1}$. In conclusion, the vitamin $B_{12}$ measurement failed, because the extracellular vitamins in the microalgal cultures were too low for detection and quantification due to a rapid uptake by the microalgal cells.

# Chapter 5

# 5 The marine diatom *Thalassiosira rotula* assembles a stable and reproducible microbiome

## 5.1 Introduction

The objective of this chapter was to investigate the establishment of microbiomes on the diatom *T. rotula*.

Diatoms are eukaryotic microalgae that are responsible for about 20% of the global photosynthesis (Malviya et al., 2016) and thus largely contribute to the oceanic biogeochemical cycle (Falkowski et al., 2008). However, the health and performance of these environmentally important photosynthetic organisms (Armbrust, 2009) depend on the association with bacteria (Amin et al., 2012). It is therefore important that microalgae are colonized by appropriate bacteria, because these bacteria may perform several advantageous tasks for the microalgal host, such as the remineralization of the released carbon compounds to make them available again for primary production (Seymour et al., 2017). Furthermore, bacteria provide a variety of growth-enhancing micronutrients to the algal host such as B-vitamins (Croft et al., 2005; Cruz-López et al., 2018), phytohormones (Amin et al., 2015) and increase the bio-availability of iron for the microalgal host (Amin et al., 2009). Therefore, the assembling of the microbiome can have a strong impact on the host fitness and performance.

The host microbiome can be governed by both the physiology of the host and external environmental factors (Marzinelli et al., 2015). Processes guiding the microbiome establishment seem to be highly regulated in some species, decreasing inter-individual variation (Berg et al., 2016), while in other species, numerous factors contribute to inter-individual differences in the microbiome composition (Boissiere et al., 2012). The surrounding water column will be the first defining factor for the potential settlement of the bacteria on the algal host. These bacteria will then be selectively attracted by the interactions with the host and primary settled bacteria (Smith et al., 2015).

However, not only the interactions between host and primary settled bacteria can determine the shape of the microbiome, but also the biotic interactions between bacteria can influence the assembling of the microbiome (Robinson et al., 2010). Although the impact of distinctions in environmental bacterial community composition on the establishment of microbiomes has already been investigated in some terrestrial and aquatic studies (Berg et al., 2016; Callens et al., 2018), there are yet only few data available on the assembly of microbiomes on microalgae.

To fill the gap in knowledge about microbiome assembling on microalgae, the assembly of microbiomes was addressed on the marine diatom *T. rotula*, which belongs to the ubiquitous and ecological important genus *Thalassiosira* (Malviya et al., 2016). The establishment of microbiomes was investigated by co-culturing an axenic *T. rotula* strain together with compositionally different bacterial source communities that have been detached from various diatom species. To study the assembling of microbiomes, the experiment described in chapter 4 was repeated. Therewith, the associated bacterial community composition of the donor diatom species (hereafter *bacterial source communities*) and of the acceptor diatom *T. rotula* (hereafter *microbiomes*) were analysed in two independent experiments. The co-cultivation of the first experiment (Experiment I) was performed in triplicates while the triplicates of each treatment were pooled to one sample before 16S rRNA sequencing. In contrast, the co-cultivation as well as the 16S rRNA sequencing of the second experiment (Experiment II) were performed with each replicate individually. The repetition of experiments was done to investigate whether the assembly of the microbiomes associated with the diatom *T. rotula* were reproducible and stable in both independent experiments. Additionally, the bacterial source communities of a seawater sample and of another *T. rotula* (A17) culture were used as inoculum to study the assembly of the microbiome with a bacterial source community with a high diversity of bacteria and from another *T. rotula* strain isolated in the same sampling location but different season.

The first aim of this chapter was to confirm the results of Experiment I. In Experiment I (Chapter 4) it was shown that the bacterial community compositions of the acceptor microbiomes resemble each other while the bacterial community compositions

of the bacterial source communities were distinct to each other. Based on the previous results of chapter 4, it was hypothesized that all newly assembled acceptor cultures harbour similar microbiomes whereas that the donor cultures harbour unique and specific bacterial source communities. In addition, the study aimed to test whether: (A) The newly formed microbiomes on the *T. rotula* acceptor resemble the original *T. rotula* acceptor microbiomes even when inoculated with high diverse seawater bacterial source communities. (B) The *T. rotula* acceptor assembles a core microbiome that has high overlap of OTUs across the newly shaped acceptor microbiomes.

## 5.2 Results and Discussion

### 5.2.1 Co-culture of the axenic *T. rotula* with distinct donor bacterial communities

The co-cultures of the axenic and vitamin-free *T. rotula* acceptor cultures together with the different inoculated bacterial source communities revealed that the growth of the diatom could be restored with each of the bacterial source communities (Figure 5.1). However, the axenic *T. rotula* acceptor diatoms in co-cultures with bacterial source communities obtained from different diatoms or seawater reached significantly higher RFU values after 96 h than with the original *T. rotula* bacterial source communities (Figure 5.1, Table 5.1). In contrast, the axenic acceptor cultures showed stagnant growth during the entire experiment duration (Figure 5.1). Therefore, the growth restoration of the axenic and vitamin-free *T. rotula* acceptor cultures was independently demonstrated in two co-culture experiments (Experiment II: Figure 5.1; Experiment I: Figure 4.1). The growth restoration of the axenic *T. rotula* axenic acceptor cultures revealed that all newly assembled microbiomes on the *T. rotula* acceptor diatoms host bacteria, which are able to provide the algal partner with sufficient amounts of vitamins to maintain the growth and fitness of the alga within 96 h in vitamin-free artificial seawater medium.

Table 5.1. Pairwise comparison of growth of *Thalassiosira rotula* acceptor cultures after 96 h (4 d) inoculated with the original *T. rotula* versus bacterial source communities obtained from different diatom species and seawater (Tukey HSD). Bold numbers indicate significant results (< 0.05).

| Pairwise comparison after 96 h of growth of *T. rotula* acceptor cultures inoculated with bacterial source communities of: | *p*-value (Tukey HSD) |
|---|---|
| *T. rotula* vs *C. closterium* | 0.992 |
| *T. rotula* vs *D. brightwellii* | 0.446 |
| *T. rotula* vs *T. pseudonana* | 0.464 |
| *T. rotula* vs *T. rotula* (A17) | **< 0.001** |
| *T. rotula* vs *T. weissflogii* | 0.068 |
| *T. rotula* vs Seawater | **< 0.001** |

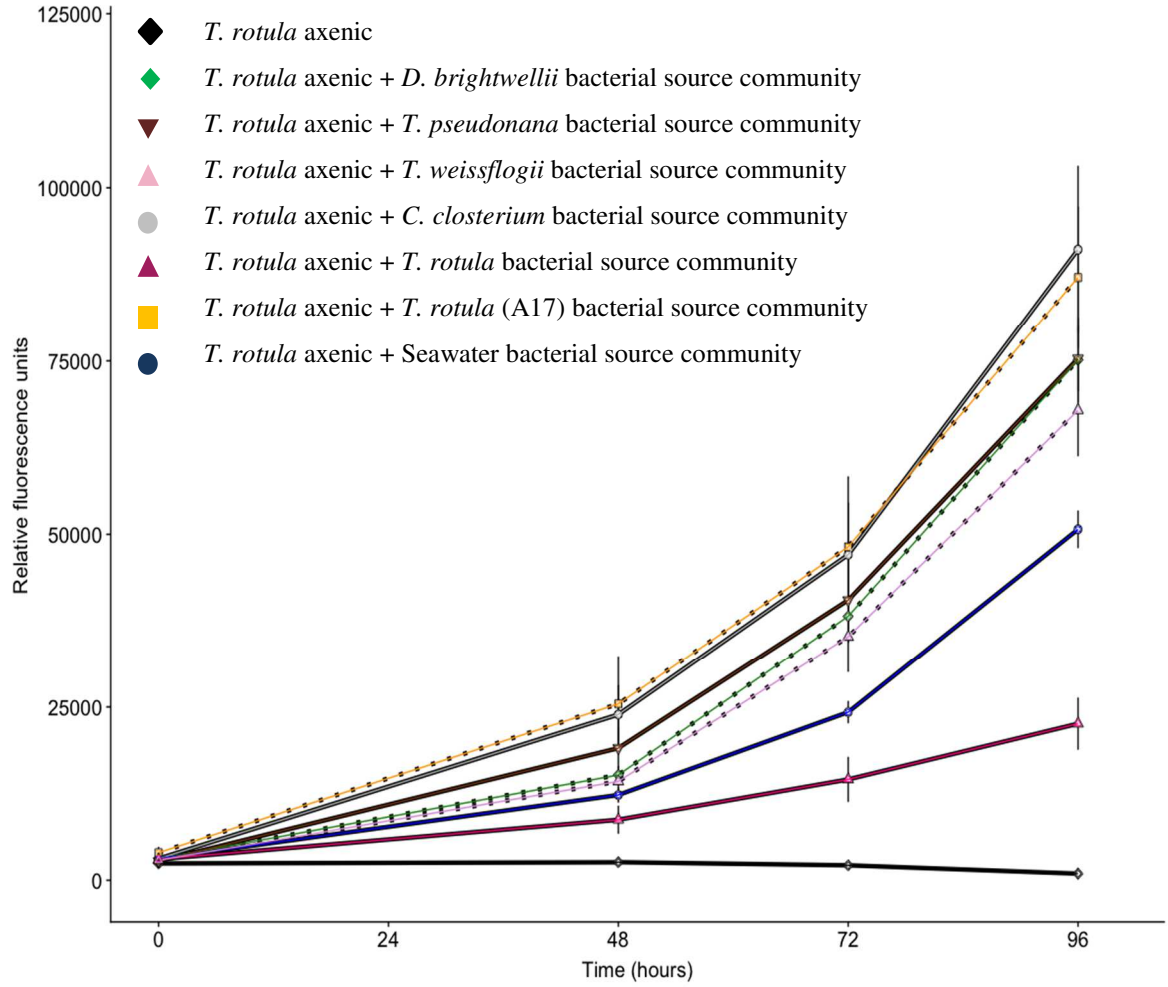



Figure 5.1. Growth of axenic *Thalassiosira rotula* acceptor cultures under absence of vitamins (black diamond, solid line) in co-culture with different inoculated bacterial source communities obtained from *Ditylum brightwellii* (green diamond, dotted line), *T. pseudonana* (brown triangle, solid line), *T. weissflogii* (plum triangle, dotted line), *Cylindrotheca closterium* (grey circle, solid line), *T. rotula* (deeppink triangle, solid line), *T. rotula* (A17) (orange square, dotted line) and seawater (blue circle, solid line). Algal growth was determined by relative fluorescence units (RFU) (n = 3). Error bars depict ± 1 SD.

### 5.2.2 Composition of the donor bacterial communities and the microbiomes on the *T. rotula* acceptor

The bacterial community composition and diversity were investigated in six different donor bacterial source communities and newly assembled microbiomes on the *T. rotula* acceptor by 16S rRNA gene amplicon sequencing. The sequencing was done separately for the samples from Experiment I (Figure 4.1) with one replicate and Experiment II (Figure 5.1) with three replicates (four samples per treatment in total). To investigate the bacterial community composition of both experiments together, the results were pooled prior to sequence data processing and bacterial community analysis. The clustering of pooled bacterial 16S rRNA gene amplicons was done using a bootstrap cut-off of 80% in RDP. Overall, more than 5 million raw reads were generated in both sequencing runs together, whereof roughly 1.6 million were affiliated to the domain of Bacteria. The following analysis resulted in the identification of 27 - 4406 OTUs in the microbiomes on the *T. rotula* acceptor and donor bacterial source communities (Figure 5.2) and a total of 6296 OTUs were identified. Rarefaction analysis of the samples of the bacterial source communities as well of the microbiomes on the *T. rotula* acceptor diatoms showed a variable sequencing depth (Figure 5.2). Therefore, all samples of both experiments were normalized on the median sequencing depth to reduce the variability between samples caused by the bias of NGS sequencing. The axenic controls of the *T. rotula* acceptor showed flatter curves in the rarefaction curve diagram compared to all other treatments (Figure 5.2, black line) as only chloroplast and mitochondrial sequences and some negligible bacterial sequences were read during sequencing. However, no bacteria were detectable by epifluorescence microscopy after DAPI staining before and after the experiment, indicating sterile conditions throughout the whole experiment.

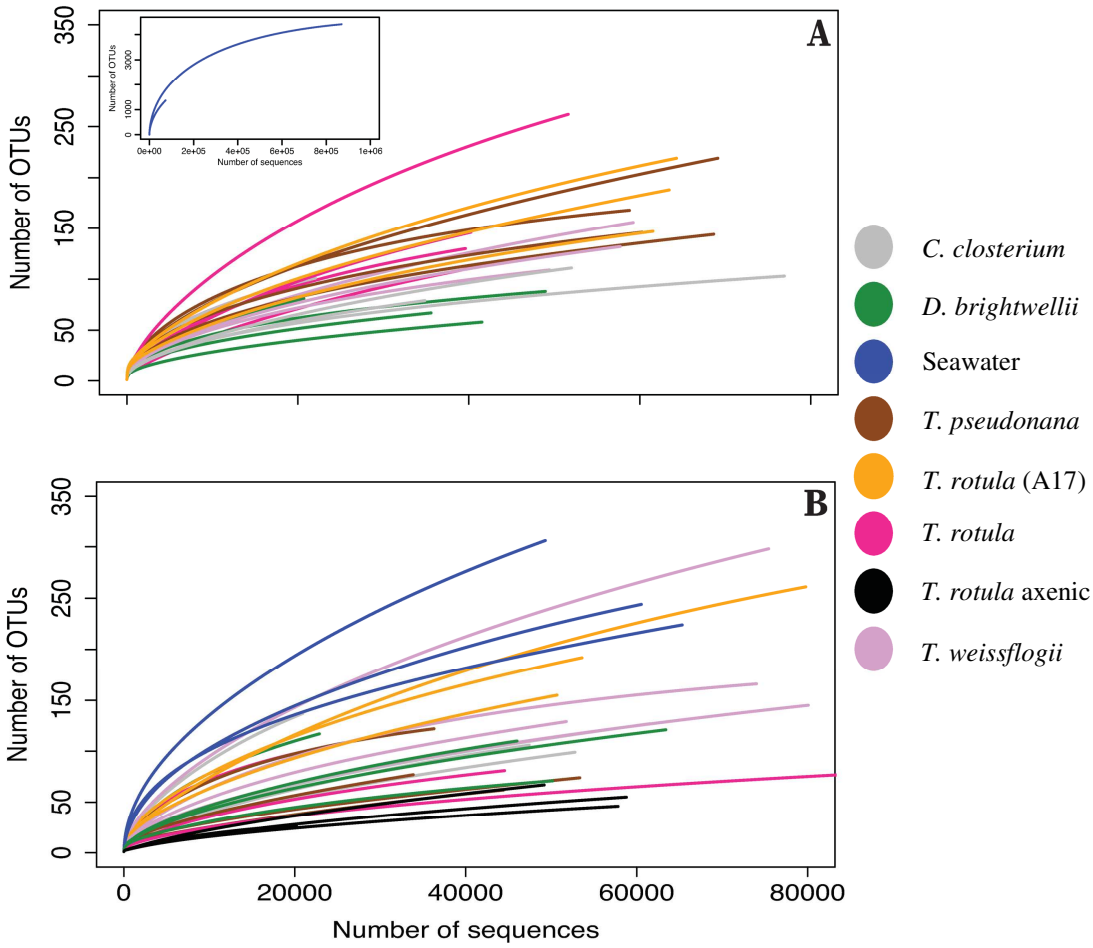



Figure 5.2. Rarefaction curves for each of the sequenced bacterial community samples of the Experiment I (Chapter 4) and Experiment II (with bacterial OTUs assigned at an 80% confidence cut-off). The bacterial source community samples are denoted as (A) and the *T. rotula* acceptor samples as (B). Inset: Rarefaction curve of the two seawater bacterial source communities.

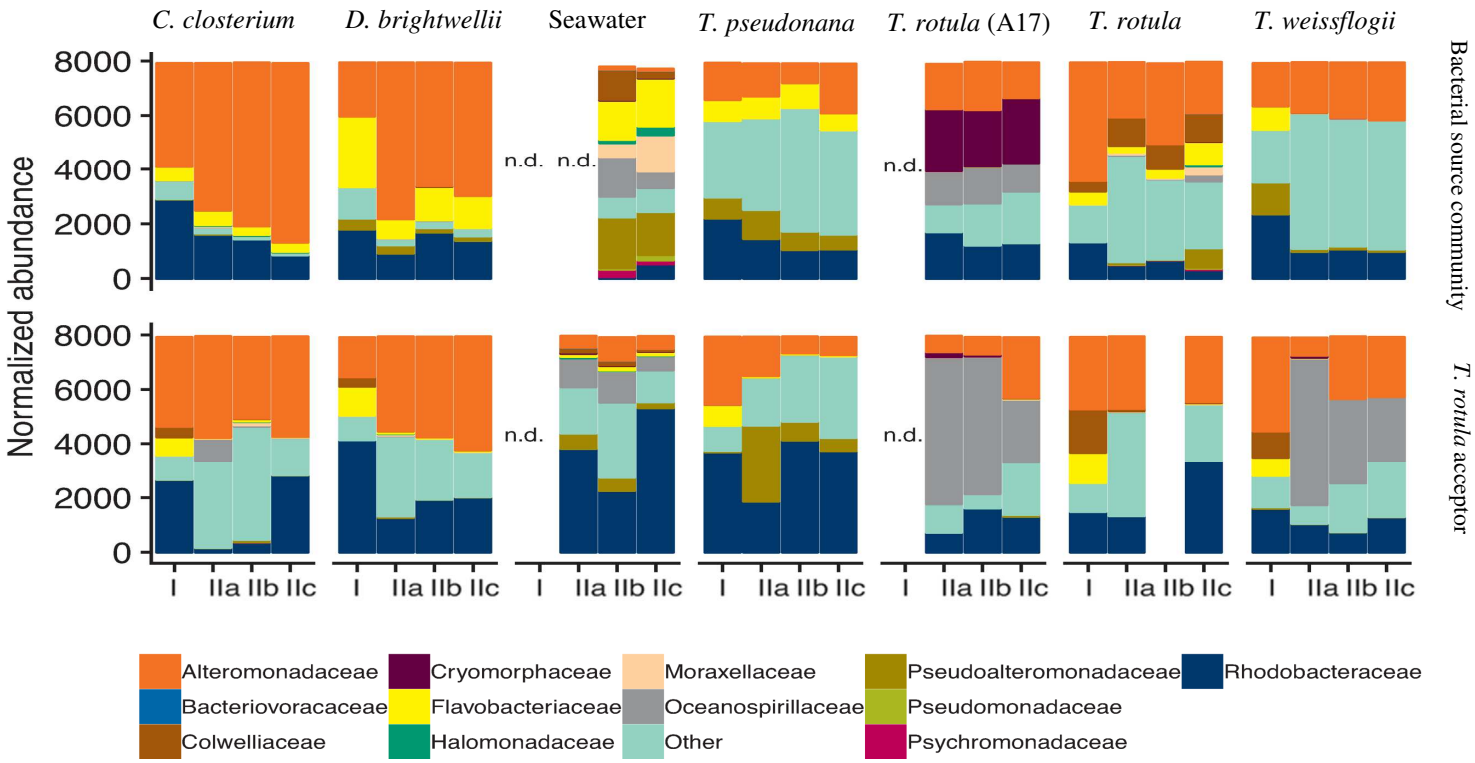



Figure 5.3. Phylogenetic composition of bacterial source communities obtained from different donor diatoms and seawater (top row) and the microbiomes of the *Thalassiosira rotula* acceptor culture inoculated with the corresponding diatom or seawater bacterial source communities (bottom row). The normalized abundance of OTUs at the family level was analysed with R using the Phyloseq package (McMurdie and Holmes, 2013). The pooled samples of Experiment I (described in Chapter 4) are indicated as (I). The individual samples from Experiment II are indicated as (IIa-c). n.d.: not determined.

The phylogenetic composition of the bacterial source communities on the donor diatoms and the newly assembled microbiomes on the *T. rotula* acceptor were visualized in stacked bar plots showing the normalized abundance at the family level (Fig. 5.3). The most abundant OTUs in the donor bacterial source communities and the microbiomes on the *T. rotula* acceptor belong to the bacterial families *Alteromonadaceae* and *Rhodobacteraceae* (Fig. 5.3). Significant differences in the bacterial community structures were apparent between the donor bacterial source communities and microbiomes on the *T. rotula* acceptor (ANOSIM: R = 0.154, $p$ = 0.001) (Fig. 5.4). Subsequently, the donor bacterial source communities were analysed individually, revealing significant differences between the donor cultures (ANOSIM: R = 0.751, $p$ = 0.001) (Fig. 5.4). The significant differences between all donor bacterial source communities imply that the donor diatoms harboured a stable, unique and specific bacterial community in both experiments. However, no significant differences were observed between the different microbiomes on the *T. rotula* acceptor (ANOSIM: R = 0.133, $p$ = 0.084) (Fig. 5.4), indicating that the microbiomes had become more similar to each other regarding the bacterial community composition even though they had been obtained from significantly different bacterial source communities. Thus, Experiment II confirmed the results from Experiment I and consequently the hypothesis was accepted that all newly assembled microbiomes on the *T. rotula* acceptor are composed of similar bacterial taxa whereas the bacterial source communities are more composed of unique and specific bacterial taxa. In particular, also the co-culture with the bacterial source community obtained from seawater led statistically to the same bacterial community compositions as observed for all other newly assembled microbiomes on the *T. rotula* acceptor. The fact that the inoculation of the seawater bacterial source communities also led statistically to the same microbiomes on the *T. rotula* acceptor is remarkable, because the seawater bacterial source communities contained a vast diversity of bacteria with up to 4406 OTUs compared to all other donor bacterial source communities (Fig. 5.2). Overall, these results showed that the newly assembled microbiomes were no longer significantly different from each other when the axenic *T. rotula* acceptor was inoculated with significantly different bacterial source communities from diatoms or seawater. Furthermore, it was observed that all newly assembled microbiomes on the *T. rotula* acceptor are more similar to the original *T. rotula* bacterial source communities than to

the inoculated bacterial source communities (Fig. 5.4). Therefore, the second hypothesis was accepted, because the newly assembled microbiomes on the *T. rotula* acceptor were also *T. rotula*-specific when inoculated with the highly diverse seawater bacterial source community.

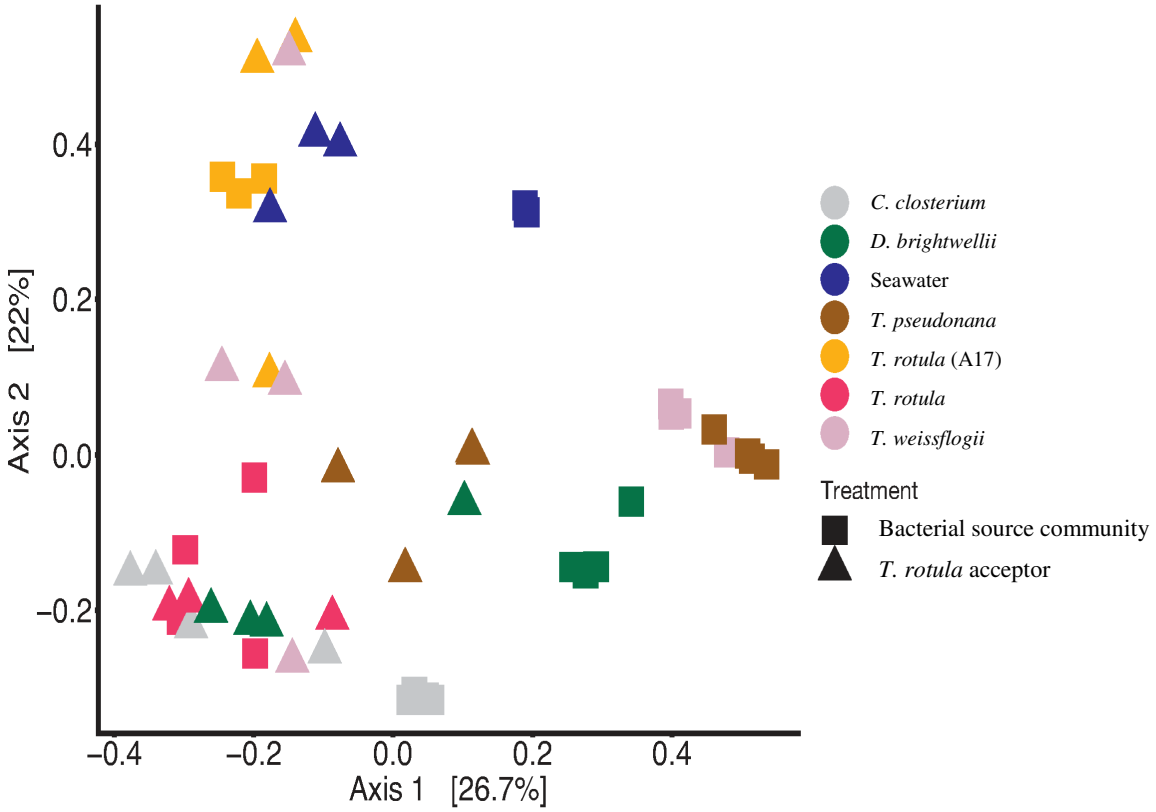



Figure 5.4. Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity of the pooled samples of Experiment I (Chapter 4) and Experiment II. The bacterial source communities are denoted as squares and the newly established microbiomes on the *T. rotula* acceptor as triangle. The different colours describe the origin of the bacterial source communities or the newly established microbiomes on the *T. rotula* acceptor.

### 5.2.3 The *Thalassiosira rotula* core microbiome

The similarity and *T. rotula*-specificity of the newly assembled microbiomes suggests the existence of OTUs with a high degree of overlap across all acceptor microbiomes. To explore the similarity and *T. rotula*-specificity of the newly assembled microbiomes more directly, the presence of core microbiome members within the newly assembled microbiomes on the *T. rotula* acceptor was determined. For this purpose, 10 OTUs were selected out of 6296 OTUs, as the similarity percentage analysis (SIMPER) found that these OTUs contributed most to the differences between the acceptor microbiomes and the bacterial source communities (Table 5.2 & 5.3). Furthermore, these 10 OTUs accounted for 82 ± 6% of the total relative abundance of the bacterial community composition in the different acceptor microbiomes and original *T. rotula* bacterial source communities.

Overall, three different scenarios were identified how the 10 OTUs were present in the acceptor microbiomes and how they differed in the presence and frequency in the corresponding bacterial source communities. The first scenario is that an OTU was abundant in the bacterial source communities and hence also settled abundantly in the acceptor microbiomes (Table 5.2 & 5.3). Thus, the first scenario might imply that the OTU was equally important for the donor species of the bacterial source community and the acceptor diatom species. In the second scenario, an OTU was scarcely present in the bacterial source communities and was nonetheless abundantly found in the acceptor microbiomes (Table 5.2 & 5.3). Therefore, the second scenario might indicate that the OTU was more important for the acceptor than for the donor diatom. The third scenario is that an OTU was abundant in the bacterial source communities and nonetheless settled scarcely in the acceptor microbiomes (Table 5.2 & 5.3). Hence, the third scenario might denote that the OTU was more important for the donor than for the acceptor diatom species.

The *T. rotula* acceptor microbiomes resulting from co-cultivation of the axenic *T. rotula* acceptor diatoms and the original *T. rotula* bacterial source communities resembled the original bacterial source communities of *T. rotula* based on the 10 OTUs (Table 5.2). The reassembling of the original *T. rotula* bacterial source communities can

be supported by the results of two-sample t-tests, which revealed that all 10 OTUs were present in the same abundance in the *T. rotula* bacterial source communities and the *T. rotula* acceptor microbiomes (Two sample t-test: $p > 0.2$). The mirroring of the inoculated *T. rotula* bacterial source communities and the *T. rotula* acceptor microbiomes confirms the success of the method as it was possible to dissociate the bacterial source community from the diatom *T. rotula* and to re-establish this community in the *T. rotula* acceptor microbiomes. The *T. rotula* (A17) acceptor microbiomes resulting from co-cultivation of the axenic *T. rotula* acceptor diatoms and the bacterial source communities of *T. rotula* (A17) resembled also the original *T. rotula* acceptor microbiomes based on the 10 representative OTUs (Table 5.2). The only major difference was observed for OTU 13 (*Marinomonas* sp.), which was significantly more present in the *T. rotula* (A17) acceptor microbiomes compared to the original *T. rotula* acceptor microbiomes (Table 5.4). Thus, OTU 13 can be categorized in the above described second scenario and the results suggest that OTU 13 (*Marinomonas* sp.) settles on the diatom host if available and consequently might indicate the significance of this OTU for the original *T. rotula* host.

Based on the 10 OTUs, the acceptor microbiomes, resulting from co-cultures with bacterial source communities obtained from diatoms of the same genus (*T. pseudonana* & *T. weissflogii*) or different genera (*D. brightwellii* & *C. closterium*), shaped a similar bacterial community composition as present in the original *T. rotula* acceptor microbiomes (Table 5.2 & 5.3). Only the relative abundances of OTU 7 and 29 differed significantly when comparing the acceptor microbiomes resulting from co-cultures with bacterial source communities of distinct diatom species and the original *T. rotula* acceptor microbiomes (Table 5.4). Notably, also the seawater acceptor microbiomes resulting from co-cultivation of the axenic *T. rotula* acceptor diatoms and the seawater bacterial source communities established a similar bacterial community composition as the original *T. rotula* acceptor microbiomes (Table 5.3). The only exceptions were the OTU 7 and 13 which were significantly less or more present on the seawater acceptor microbiomes than the original *T. rotula* acceptor microbiomes.

Overall, the results revealed similar allocations of the 10 OTUs in the different acceptor microbiomes. The only significant distinctions between the original *T. rotula*

acceptor microbiomes and the microbiomes assembled of different bacterial source communities were caused by the OTUs 7, 13 and 29. Therefore, the repeated co-culture experiments with the axenic *T. rotula* acceptor diatoms and the compositionally different bacterial source communities resulted in a stable and reproducible assembling of the 10 OTUs in the acceptor microbiomes, most similar to the bacterial community composition of the original *T. rotula* microbiomes. Furthermore, the 10 OTUs contributed more than 80% to the relative abundance of the acceptor microbiomes and are therefore designated as the core microbiome of the diatom *T. rotula*. However, even if not all 10 OTUs were present in each of the acceptor microbiomes, these 10 OTUs can be described as the core microbiome due to their high abundance in the acceptor microbiomes and their responsibility for the differentiation between bacterial source communities and acceptor microbiomes.

A core microbiome is typically defined to have a high degree of overlapping members in different microbiomes of the same species (Turnbaugh et al., 2009). Here, it was shown that the diatom species *T. rotula* maintains a core microbiome with 10 OTUs that have a high degree of overlap in 25 assembled microbiomes resulting from different inoculated bacterial source communities and generated in two different experiments. The method used in this chapter to define the core microbiome members has the advantage that not only the presence and absence of OTUs is considered, but also the abundance of existing OTUs in the microbiomes. In contrast, the Venn analysis, typically used to determine core microbiomes (Shade and Handelsman, 2012), weighs all observed OTUs equally, regardless of their representation in the community. Based on the large overlap of the 10 OTUs in the different assembled microbiomes in two independent experiments, the third hypothesis that *T. rotula* assembles a stable and reproducible core microbiome with a high overlap across the newly shaped acceptor microbiomes can be accepted.

Using the co-culture system of the axenic *T. rotula* acceptor diatoms and the different inoculated bacterial source communities, the assembling of the microbiome of the diatom *T. rotula* was investigated. The bacterial composition of the different newly established acceptor microbiomes was reproducibly similar, but distinct from their respective bacterial source communities except for the original *T. rotula* bacterial source

community. The reproducible similarity of the acceptor microbiomes suggested deterministic shaping of the *T. rotula* microbiome that was largely independent of the diversity of the inoculated bacterial communities. The results also showed that the similarity of all newly established acceptor microbiomes with the original *T. rotula* bacterial source communities indicates a contribution of the host niche to the shape of the *T. rotula* microbiome. A possible explanation for the niche separation might be the colonization of some bacteria that were more attracted and supported by the *T. rotula* surface for metabolic and nutritional reasons than of the surfaces of the other diatom species from the same and different genera. An example for the preference of a bacterial species towards a diatom's surface over another could be the OTU 13, *Marinomonas* sp., which was significantly more abundant in the acceptor microbiomes than in the corresponding bacterial source communities. Furthermore, diatom exudates such as polysaccharides and DMSP attract heterotrophic bacteria (Bell and Mitchell, 1972; Seymour et al., 2010) and are described to be diatom species-specific (Hahnke et al., 2013; Becker et al., 2014). Hence, the nature of the diatoms and their released polysaccharides might strongly determine the bacterial microbiome assembling (Grossart et al., 2005; Sapp et al., 2007). Besides the response to nutritional factors, another possible factor that might determine the specificity of the acceptor microbiomes could be that specific traits of the diatom *T. rotula* direct the intrinsic microbiome assembling. For instance, inhibitory compounds associated with living surfaces (Saha et al., 2011; Lachnit et al., 2013) can selectively influence the microbiome assembly on a host. Diatoms have also been shown to produce antimicrobial compounds such as fatty acids, esters (Lebeau and Robert, 2003) and polyunsaturated aldehydes (Wichard et al., 2007) that can have an effect on the bacterial community composition and structure and aim to defend themselves against unwanted and/or harmful bacteria (Amin et al., 2012).

In conclusion, all newly shaped microbiomes on the diatom *T. rotula* harbour a similar bacterial community composition, which was dominated by the 10 identified bacterial core members (OTUs) that account for a total relative abundance of more than 80%. The similarity of the newly established microbiomes in all treatments during both experiments is suggesting a stable and reproducible microbiome assembling on the diatom *T. rotula*. The processes that guide the microbiome establishment on the diatom

*T. rotula* seem to be highly regulated, decreasing inter-individual variation between the different treatments, replicates and experiments. The results of the microbiome assembly on *T. rotula* lend support to host factors, more than the environmental bacterial diversity, as dominant contributors in shaping the microbiome composition. Although the results do not reveal which factors are at play, this study showed the host-specific microbiome assembly on an environmentally important diatom species for the first time.

Table 5.2. Relative abundances of the 10 OTUs most responsible for the differences between the acceptor microbiomes and the bacterial source communities of *T. rotula* (Original), *T. rotula* (A17), *T. pseudonana* and *T. weissflogii.* The OTUs are ordered according to their percentage cumulative contribution (SIMPER, Cumsum) for the differences between the acceptor microbiomes and the bacterial source communities. The green or rather blue bars indicate individually the relative abundance of the 10 OTUs in all replicates of the different bacterial source communities or rather acceptor microbiomes.

| | OTU | Cumsum | Bacterial species | Relative abundance (%) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *T. rotula* | | | *T. rotula* (A17) | | | *T. pseudonana* | | | | *T. weissflogii* | | | |
| | | | | I | II a | II c | II a | II b | II c | I | II a | II b | II c | I | II a | II b | II c |
| Bacterial source community | 7 | 0.1356 | *Alteromonas* sp. | 51.14 | 24.06 | 36.16 | 14.18 | 14.67 | 11.92 | 0.00 | 0.01 | 0.03 | 0.03 | 2.96 | 4.46 | 4.87 | 4.22 |
| | 8 | 0.2564 | unclassified | 15.83 | 46.26 | 35.88 | 6.80 | 10.39 | 12.91 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.01 | 0.00 |
| | 12 | 0.3448 | *Alteromonas* sp. | 2.24 | 0.06 | 0.03 | 3.73 | 3.88 | 1.66 | 16.42 | 14.73 | 8.63 | 22.11 | 16.39 | 17.85 | 19.73 | 21.36 |
| | 13 | 0.4162 | *Marinomonas* sp. | 0.00 | 0.00 | 0.03 | 9.31 | 11.48 | 8.87 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.03 |
| | 11 | 0.471 | *Sulfitobacter* sp. | 9.60 | 4.00 | 5.58 | 0.00 | 0.05 | 0.00 | 0.09 | 5.50 | 4.25 | 4.69 | 0.05 | 0.04 | 0.04 | 0.05 |
| | 17 | 0.5247 | unclassified | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 31.54 | 40.79 | 55.31 | 46.40 | 0.00 | 0.00 | 0.01 | 0.03 |
| | 27 | 0.5773 | unclassified | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 18.17 | 57.95 | 55.06 | 54.63 |
| | 29 | 0.6227 | *Sulfitobacter* sp. | 2.56 | 0.26 | 0.13 | 0.00 | 0.00 | 0.00 | 13.93 | 0.58 | 0.43 | 0.52 | 17.55 | 1.66 | 2.18 | 1.90 |
| | 33 | 0.6645 | *Croceibacter* sp. | 5.64 | 1.81 | 3.44 | 0.00 | 0.00 | 0.00 | 9.47 | 9.75 | 11.14 | 7.44 | 10.54 | 0.00 | 0.01 | 0.00 |
| | 2 | 0.7041 | *Pseudoalteromonas* sp. | 0.04 | 1.02 | 0.25 | 0.00 | 0.01 | 0.00 | 9.43 | 13.11 | 8.22 | 6.62 | 14.45 | 1.30 | 1.26 | 1.11 |
| | Total reads | | | 7941 | 7950 | 7899 | 7886 | 7962 | 7933 | 7921 | 7907 | 7903 | 7888 | 7915 | 7946 | 7932 | 7941 |
| *T. rotula* acceptor | 7 | 0.1356 | *Alteromonas* sp. | 28.56 | 31.58 | 29.44 | 5.01 | 5.38 | 23.89 | 26.03 | 14.92 | 2.76 | 1.12 | 42.48 | 5.18 | 23.59 | 21.71 |
| | 8 | 0.2564 | unclassified | 12.12 | 46.70 | 26.07 | 10.24 | 4.92 | 14.50 | 9.30 | 21.69 | 14.40 | 16.56 | 11.88 | 6.90 | 19.37 | 16.15 |
| | 12 | 0.3448 | *Alteromonas* sp. | 3.32 | 0.00 | 0.03 | 1.39 | 1.76 | 3.68 | 4.30 | 2.80 | 4.66 | 6.85 | 0.18 | 2.36 | 4.46 | 5.27 |
| | 13 | 0.4162 | *Marinomonas* sp. | 0.00 | 0.05 | 0.00 | 51.04 | 49.01 | 23.57 | 0.00 | 0.00 | 0.03 | 0.00 | 0.00 | 51.03 | 37.50 | 28.76 |
| | 11 | 0.471 | *Sulfitobacter* sp. | 7.93 | 13.22 | 38.70 | 0.03 | 0.01 | 1.24 | 0.21 | 14.06 | 23.21 | 25.15 | 6.60 | 0.01 | 0.50 | 0.95 |
| | 17 | 0.5247 | unclassified | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.44 | 0.01 | 0.00 | 15.77 | 20.84 | 0.00 | 0.00 | 0.11 | 0.05 |
| | 27 | 0.5773 | unclassified | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 6.90 | 0.54 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.73 | 7.59 |
| | 29 | 0.6227 | *Sulfitobacter* sp. | 2.61 | 0.54 | 1.10 | 0.00 | 0.00 | 8.59 | 7.41 | 4.11 | 14.52 | 7.38 | 7.10 | 0.00 | 5.39 | 10.31 |
| | 33 | 0.6645 | *Croceibacter* sp. | 13.80 | 0.14 | 0.34 | 0.00 | 0.01 | 0.05 | 9.29 | 0.62 | 0.38 | 0.52 | 8.27 | 0.00 | 0.01 | 0.00 |
| | 2 | 0.7041 | *Pseudoalteromonas* sp. | 0.00 | 0.00 | 0.00 | 0.01 | 0.01 | 0.70 | 0.62 | 35.28 | 8.61 | 6.10 | 0.73 | 0.11 | 0.19 | 0.11 |
| | Total reads | | | 7919 | 7933 | 7910 | 7961 | 7911 | 7908 | 7924 | 7929 | 7935 | 7912 | 7898 | 7896 | 7936 | 7918 |

Table 5.3. Relative abundances of the 10 OTUs most responsible for the differences between the acceptor microbiomes and the bacterial source communities of *T. rotula* (Original), *D. brightwellii*, *C. closterium* and seawater. The OTUs are ordered according to their percentage cumulative contribution (SIMPER, Cumsum) for the differences between the acceptor microbiomes and the bacterial source communities. The green or rather blue bars indicate individually the relative abundance of the 10 OTUs in all replicates of the different bacterial source communities or rather acceptor microbiomes.

| | OTU | Cumsum | Bacterial species | Relative abundance (%) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *T. rotula* | | | *D. brightwellii* | | | | *C. closterium* | | | | Seawater | | |
| | | | | I | II a | II c | I | II a | II b | II c | I | II a | II b | II c | II a | II b | II c |
| Bacterial source community | 7 | 0.1356 | *Alteromonas* sp. | 51.14 | 24.06 | 36.16 | 8.91 | 19.08 | 18.52 | 16.48 | 35.70 | 46.47 | 53.95 | 61.33 | | 0.21 | 0.14 |
| | 8 | 0.2564 | unclassified | 15.83 | 46.26 | 35.88 | 0.00 | 0.00 | 0.11 | 0.04 | 0.04 | 0.00 | 0.04 | 0.01 | | 0.00 | 0.00 |
| | 12 | 0.3448 | *Alteromonas* sp. | 2.24 | 0.06 | 0.03 | 14.98 | 51.11 | 38.24 | 43.27 | 10.31 | 20.60 | 19.20 | 19.17 | | 0.01 | 0.01 |
| | 13 | 0.4162 | *Marinomonas* sp. | 0.00 | 0.00 | 0.03 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | | 0.26 | 0.12 |
| | 11 | 0.471 | *Sulfitobacter* sp. | 9.60 | 4.00 | 5.58 | 0.10 | 1.32 | 0.54 | 1.34 | 21.58 | 15.20 | 15.13 | 8.26 | | 0.05 | 0.30 |
| | 17 | 0.5247 | unclassified | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 2.10 | 0.00 | 0.00 | 0.03 | | 0.00 | 0.00 |
| | 27 | 0.5773 | unclassified | 0.00 | 0.00 | 0.00 | 0.19 | 0.09 | 0.11 | 0.08 | 0.00 | 0.00 | 0.00 | 0.00 | | 0.00 | 0.00 |
| | 29 | 0.6227 | *Sulfitobacter* sp. | 2.56 | 0.26 | 0.13 | 15.90 | 1.16 | 3.81 | 2.45 | 5.60 | 4.50 | 2.67 | 2.31 | | 0.00 | 0.03 |
| | 33 | 0.6645 | *Croceibacter* sp. | 5.64 | 1.81 | 3.44 | 32.46 | 8.29 | 15.04 | 14.11 | 5.67 | 6.23 | 3.93 | 4.13 | | 0.01 | 0.00 |
| | 2 | 0.7041 | *Pseudoalteromonas* sp. | 0.04 | 1.02 | 0.25 | 4.84 | 3.82 | 2.07 | 1.70 | 0.24 | 0.42 | 0.04 | 0.01 | | 22.88 | 19.74 |
| | Total reads | | | 7941 | 7950 | 7899 | 7932 | 7934 | 7932 | 7930 | 7916 | 7917 | 7933 | 7914 | | 7784 | 7710 |
| *T. rotula* acceptor | 7 | 0.1356 | *Alteromonas* sp. | 28.56 | 31.58 | 29.44 | 11.15 | 40.38 | 41.13 | 47.24 | 38.61 | 44.56 | 35.97 | 43.43 | 3.30 | 5.15 | 1.99 |
| | 8 | 0.2564 | unclassified | 12.12 | 46.70 | 26.07 | 10.24 | 35.68 | 27.70 | 20.08 | 9.88 | 33.79 | 49.91 | 16.58 | 15.25 | 30.98 | 11.23 |
| | 12 | 0.3448 | *Alteromonas* sp. | 3.32 | 0.00 | 0.03 | 6.72 | 1.57 | 4.20 | 3.76 | 1.59 | 0.57 | 0.21 | 1.32 | 1.13 | 3.80 | 2.76 |
| | 13 | 0.4162 | *Marinomonas* sp. | 0.00 | 0.05 | 0.00 | 0.00 | 0.05 | 0.00 | 0.01 | 0.00 | 0.03 | 0.00 | 0.01 | 4.86 | 5.92 | 3.37 |
| | 11 | 0.471 | *Sulfitobacter* sp. | 7.93 | 13.22 | 38.70 | 1.37 | 1.23 | 2.01 | 1.55 | 8.22 | 0.21 | 2.77 | 32.07 | 0.06 | 0.09 | 0.13 |
| | 17 | 0.5247 | unclassified | 0.00 | 0.00 | 0.00 | 0.14 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.05 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 27 | 0.5773 | unclassified | 0.00 | 0.00 | 0.00 | 0.00 | 0.03 | 0.03 | 0.00 | 0.00 | 0.00 | 0.10 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 29 | 0.6227 | *Sulfitobacter* sp. | 2.61 | 0.54 | 1.10 | 39.93 | 8.45 | 14.73 | 12.88 | 16.12 | 0.03 | 1.06 | 2.73 | 0.15 | 0.32 | 0.18 |
| | 33 | 0.6645 | *Croceibacter* sp. | 13.80 | 0.14 | 0.34 | 13.13 | 0.49 | 0.45 | 0.39 | 8.14 | 0.00 | 0.10 | 0.04 | 0.00 | 0.00 | 0.00 |
| | 2 | 0.7041 | *Pseudoalteromonas* sp. | 0.00 | 0.00 | 0.00 | 0.03 | 0.67 | 0.04 | 0.15 | 0.04 | 0.03 | 0.96 | 0.18 | 6.92 | 6.11 | 2.71 |
| | Total reads | | | 7919 | 7933 | 7910 | 7907 | 7940 | 7927 | 7932 | 7916 | 7949 | 7910 | 7939 | 7963 | 7902 | 7944 |

Table 5.4. Pairwise comparison of the original *T. rotula* acceptor microbiomes compared with the acceptor microbiomes assembled of the bacterial source communities of *C. closterium*, *T. rotula* (A17), *T. weissflogii*, *T. pseudonana*, *D. brightwellii* and seawater (Two sample t-test). Bold numbers indicate significant results (< 0.05). NA: not analysed.

| Pairwise comparison of: | *p*-value two sample t-test | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | OTU 7 | OTU 8 | OTU 12 | OTU 13 | OTU 11 | OTU 17 | OTU 27 | OTU 29 | OTU 33 | OTU 2 |
| *T. rotula* acceptor microbiomes vs *C. closterium* acceptor microbiomes | **0.007** | 0.957 | 0.879 | 0.016 | 0.486 | 0.391 | 0.391 | 0.414 | 0.627 | 0.266 |
| *T. rotula* acceptor microbiomes vs *T. rotula* (A17) acceptor microbiomes | 0.0949 | 0.202 | 0.434 | **0.043** | 0.175 | 0.409 | 0.422 | 0.666 | 0.404 | 0.406 |
| *T. rotula* acceptor microbiomes vs *T. weissflogii* acceptor microbiomes | 0.45 | 0.277 | 0.274 | 0.072 | 0.197 | 0.223 | 0.341 | 0.139 | 0.627 | 0.152 |
| *T. rotula* acceptor microbiomes vs *T. pseudonana* acceptor microbiomes | **0.047** | 0.329 | 0.061 | 0.652 | 0.721 | 0.187 | 0.391 | **0.046** | 0.71 | 0.2 |
| *T. rotula* acceptor microbiomes vs *D. brightwellii* acceptor microbiomes | 0.573 | 0.697 | 0.115 | 0.939 | 0.192 | 0.391 | 0.181 | 0.089 | 0.846 | 0.238 |
| *T. rotula* acceptor microbiomes vs Seawater acceptor microbiomes | **0.001** | 0.487 | 0.349 | **0.023** | 0.171 | NA | NA | 0.19 | 0.402 | 0.055 |

## 5.3 Appendix: Manuscript under revision, ISME journal

# Niche-based assembly of the bacterial microbiome on the diatom *Thalassiosira rotula* is stable and reproducible

**Julian Mönnich**, Jan Tebben, Jennifer Bergemann, Rebecca J. Case, Sylke Wohlrab and Tilmann Harder

My contribution to this manuscript is denoted as follows:

- Conceived the study: **JM**, JT, RC and TH
- Isolated the diatoms and performed the experiments: **JM** and JB
- Analysed the data: **JM**, JT, TH and SW
- Wrote the manuscript: TH

- All Authors accepted the final manuscript

**Declaration on the contribution of the candidate to a multi-author article/manuscript which is included as a chapter in the submitted doctoral thesis**

**Chapter:** Niche-based assembly of the bacterial microbiome on the diatom Thalassiosira rotula is stable and reproducible

**Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):**

| | |
|---|---|
| Experimental concept and design: | ca. 25 % |
| Experimental work and/or acquisition of (experimental) data: | ca. 80 % |
| Data analysis and interpretation: | ca. 25 % |
| Preparation of Figures and Tables: | ca. 100 % |
| Drafting of the manuscript: | ca. 0 % |

**Chapter:**

**Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):**

| | |
|---|---|
| Experimental concept and design: | ca. __ % |
| Experimental work and/or acquisition of (experimental) data: | ca. __ % |
| Data analysis and interpretation: | ca. __ % |
| Preparation of Figures and Tables: | ca. __ % |
| Drafting of the manuscript: | ca. __ % |

**Chapter:**

**Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):**

| | |
|---|---|
| Experimental concept and design: | ca. __ % |
| Experimental work and/or acquisition of (experimental) data: | ca. __ % |
| Data analysis and interpretation: | ca. __ % |
| Preparation of Figures and Tables: | ca. __ % |
| Drafting of the manuscript: | ca. __ % |

Date: 26.07.19

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## Abstract

With each cell division, phytoplankton create new space for primary colonization by marine bacteria. Although this surface microenvironment is available to all planktonic bacterial colonizers, we show microbiome assembly on a cosmopolitan marine diatom to be highly specific and reproducible. While phytoplankton-bacteria interactions play fundamental roles in marine ecosystems, namely primary production and the carbon cycle, the ecological paradigm behind epiphytic microbiome assembly remains poorly understood. In a replicated and repeated primary colonization experiment, we exposed the axenic diatom *Thalassiosira rotula* to several complex and compositionally different bacterial inocula derived from phytoplankton species of varying degrees of relatedness to the axenic Thalassiosira host or natural seawater. This revealed a convergent assembly of diverse and compositionally different bacterial inocula, containing up to 2071 operational taxonomic units (OTUs), towards a stable and reproducible core microbiome. Only 4 of these OTUs already accounted for a total abundance of 60%. The microbiome was dominated by *Rhodobacteraceae* (30.5%), *Alteromonadaceae* (27.7%), *Oceanospirillales* (18.5%) which was qualitatively and quantitatively most similar to its conspecific original. These findings reject a lottery assembly model of bacterial colonization and suggest selective microhabitat filtering. This is likely due to diatom host traits such as surface properties and different levels of specialization resulting in reciprocal stable-state associations.

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# Chapter 6

# 6 General discussion

The identification of key bacteria associated with ecological important diatoms (Malviya et al., 2016) is a central step towards an understanding of the complex relationship between these two groups of microbes in the ocean. Most of our present knowledge of the interactions between diatoms and bacteria is derived from the investigations of cultured model systems, usually sourced from long-term culture collections (Amin et al., 2015; Moejes et al., 2017). The relevance of bacteria obtained long-term cultivated diatom cultures in comparison to bacterial communities associated with diatoms in the ocean is largely unknown, because it is assumed that cultivation under nutrient-rich conditions may change the composition of the microbiomes. However, not only the cultivation itself influences the microbiomes, but also the sampling location and season of microalgae can have a significant impact on microalgae-associated bacterial communities of the same species or even strain (Ajani et al., 2018). It is therefore crucial to isolate diatoms and bacteria from the field to be able to exclude changes in the diatom-associated bacterial community composition that have been caused by nutrient-rich laboratory cultivation or by different sampling locations or sampling seasons.

Based on the largely unknown effects of long-term laboratory cultivation and influences of sampling location and season on diatom-associated bacterial communities, the first aim of this thesis was to co-isolate diatoms and bacteria from a spring bloom in the German Bight of the North Sea to have recent microalgae and bacteria from the same location available for experiments **(Chapter 2)**. Therefore, four different diatom species and 200 morphological different bacteria were isolated. The isolation of diatoms and bacteria was followed by a methodical development to identify the optimal culture conditions and to determine growth and performance of the isolated diatoms and bacteria (**Chapter 2**). The four isolated diatom species served as donor and acceptor of bacterial source communities in this thesis, which implies that the bacterial communities were dissociated from the different donor diatoms and were used as bacterial inoculum for

*in vitro* studies with axenic acceptor diatoms. The marine diatom *Thalassiosira rotula* was selected as the model organism for the *in vitro* studies with diatoms and bacteria, because it belongs to the ubiquitous and ecological important genus *Thalassiosira*. The importance of the genus *Thalassiosira* for the global ocean was indicated recently by the Tara Ocean expeditions, reporting this genus to be globally one of the two most abundant diatom genera globally (Malviya et al., 2016).

Two hypotheses were derived from these initial results of **Chapter 3.** The first hypothesis is that the diatom *T. rotula* is auxotroph for B-vitamins. The second hypothesis is that the bacterial community of *T. rotula* supports the growth of vitamin depleted *T. rotula* cells by provision of B-vitamins. Both hypotheses are related to the well-recognized mutualistic interactions between microalgae and bacteria, in which bacteria supply microalgae with B-vitamins in exchange for carbon compounds (Cole, 1982; Karl, 2002; Tang et al., 2010; Wagner-Dobler et al., 2010; Kazamia et al., 2012; Grant et al., 2014; Cruz-López et al., 2018). To test these hypotheses, a medium without B-vitamins was prepared and the intracellular stored vitamins in the diatoms were depleted to investigate growth and performance of axenic and non-axenic diatoms under absence of vitamins. Growth experiments aiming to deplete intra- and extracellular stored vitamins from the medium and diatoms revealed that the growth of the axenic *T. rotula* culture completely stagnated after growing for 120 h in vitamin-free medium. At the same time, the non-axenic culture of *T. rotula* maintained its growth, indicating that the diatom *T. rotula* is auxotroph for B-vitamins and that the bacterial community is able to maintain growth of the diatom with the provision of vitamins. Hence, both hypotheses were accepted **(Chapter 3)**.

The third aim of this thesis was to describe the microbiome assembling on the diatom *T. rotula* under absence of vitamins **(Chapter 4 & 5)**. Earlier studies revealed that microalgae harbour a unique and species-specific bacterial community (Grossart et al., 2005; Amin et al., 2012; Behringer et al., 2018; Crenn et al., 2018), whereas another study showed that the sampling location and season during which microalgae were isolated had a more significant influence on the microalgae microbiome than phylogenetic affiliation (Ajani et al., 2018). Therefore, it remains unclear if the microbiome assembling of

microalgae is host-specific (Grossart et al., 2005; Crenn et al., 2018) or specified by the available environmental bacterial community (Sapp et al., 2007; Ajani et al., 2018). In order to increase the knowledge of host-specificity of the assembling of microalgae microbiomes, vitamin-free axenic cultures of the *T. rotula* acceptor were inoculated with bacterial source communities sourced from different donor diatom species in two independent experiments. Bacterial source communities of different diatom species were used, because it was shown in many previous studies that microalgae harbour unique and specific bacterial communities (Grossart et al., 2005; Amin et al., 2012; Behringer et al., 2018; Crenn et al., 2018). The aim of the inoculation of unique and specific bacterial source communities taken from different diatoms was to investigate if the diatom *T. rotula* harbours a unique and specific microbiome independent of the diversity of the inoculated bacterial source communities. It is hypothesized that all newly shaped acceptor cultures harbour similar microbiomes whereas that the donor cultures harbour unique and specific bacterial source communities as previously described for other microalgae species. Furthermore, it is hypothesised that the similarity of the newly shaped microbiomes on the diatom *T. rotula* to each other is caused by core microbiome members, which are abundantly present in the majority of the newly shaped microbiomes.

However, before the microbiome assembling was investigated, it was examined on two different diatom species whether the bacterial source communities of the donor diatoms undergo major changes in their composition during cultivation in the lab over one year **(Chapter 4)**. The comparison of the bacterial source communities of two isolated diatom species, shortly after isolation (< 1 month, origin) and after culturing in the lab for more than year (> 1 year, donor), revealed only slight differences in bacterial community composition between origin and donor bacterial communities **(Chapter 4)**. Consequently, the observation of Behringer et al. (2018) that long-term cultivation only resulted in small changes in the associated bacterial community composition can be supported with the observation on the diatoms *T. rotula* (original) and *D. brightwellii*. Thus, changes in the microbiomes of the investigated diatom species caused by long-term cultivation under nutrient-rich conditions may be excluded here due to the minor changes observed in the bacterial community composition between origin and donor bacterial communities.

After changes in the bacterial community compositions caused by long-term cultivation could be excluded, the composition of the bacterial source communities and newly established microbiomes on the *T. rotula* acceptor diatoms were analysed in two independent experiments. The first experiment was performed without replicates during sequencing, which is why the observed patterns in the composition of the bacterial source communities and acceptor microbiomes could not be statistically verified. Thus, the second experiment was done in triplicate to investigate the compositional variability of the bacterial source communities and acceptor microbiomes within the same experiment. Both experiments together did allow to investigate if the bacterial source communities and acceptor microbiomes were stable in their composition during both experiments and thus strengthening the outcomes through repeatability and reproducibility of the analysed bacterial communities. **Chapter 4** comprises the results from Experiment I while in **Chapter 5** the results from Experiment I and II were pooled and jointly analysed. To evaluate the specificity of the newly shaped microbiomes of the diatom *T. rotula*, the composition of the bacterial source communities and acceptor microbiomes was evaluated **(Chapters 4 & 5)**. The comparison of the bacterial source communities and acceptor microbiomes revealed that the bacterial source communities of the donor cultures were significantly different from each other, whereas the newly shaped acceptor microbiomes were statistically the same. These patterns were observed in the two independent experiments and within the replication in Experiment II, indicating reproducibility and repeatability of the experiments and the analysed associated bacterial communities **(Chapter 4 & 5)**.

Consequently, both experiments together revealed significant different bacterial community compositions of the analysed bacterial source communities and indicated with it that the bacterial source communities of the investigated diatom donor cultures are species-specific and thus support findings of previous studies suggesting that microalgae harbour specific bacterial communities (Grossart et al., 2005; Krohn-Molt et al., 2017; Behringer et al., 2018; Crenn et al., 2018). The observed species-specificity of the bacterial source communities of the diatoms in this thesis was probably triggered by differences in the DOC composition released by the various donor diatoms, which in turn may attract different bacteria. Indeed, Urbani et al. (2005) revealed that the monomer

composition of dissolved polysaccharides, which form that the largest fraction of DOC, depends on microalgae species. However, the quality and quantity of exudates are not only influenced by the microalgae species, but are also strongly influenced by nutrient availability during growth (reviewed in Mühlenbruch et al., 2018). The donor cultures investigated in this thesis were cultured for at least two months under vitamin-free conditions prior to the experiment. The cultivation under vitamin-free conditions might have influenced the bacterial community composition, because nutrient-limited microalgae release large amounts of polysaccharides (Wetz and Wheeler, 2007) which may serve to attract bacteria that will provide important nutrients such as iron, ammonium and vitamins (Amin et al., 2009; Sanudo-Wilhelmy et al., 2014; Amin et al., 2015). The axenic *T. rotula* culture that served as the acceptor for the different bacterial source communities in this study was also depleted of vitamins when the bacterial source communities were inoculated. The aim of the inoculation under vitamin-depletion was to attract especially bacteria that are able to provide the essential B-vitamins for *T. rotula*. Indeed, it was shown that all newly established acceptor microbiomes provided the B-vitamin auxotroph diatom *T. rotula* with the vitamins **(Chapter 3)**. However, besides the impact of abiotic factors, both the quantity and the quality of microalgae exudates also seem to be actively controlled by both bacteria (Gärdes et al., 2012) and microalgae (Amin et al., 2012).

In **Chapter 4 and 5** it was shown that all newly shaped acceptor microbiomes are more similar to the original *T. rotula* bacterial source community than to the donor cultures where the bacterial source community were obtained from. Additionally, this selection of a *T. rotula* specific microbiome was also observed with the inoculation of the bacterial source community obtained from a natural seawater sample. The bacterial diversity in the seawater bacterial source communities was much higher than in all other bacterial source communities obtained from the donor diatoms. Nonetheless, if the seawater bacterial source community was inoculated on the axenic acceptor culture, the newly established acceptor microbiome was also similar to all other acceptor microbiomes and to the original *T. rotula* bacterial source community. Consequently, the exciting similarity of the newly established acceptor microbiomes to each other and to the original *T. rotula* bacterial source community resulted most likely from a specific

DOC release of the diatom *T. rotula*. It can be suggested that *T. rotula* selectively attracts certain bacteria, likely by a release of specific DOC, because all newly established microbiomes on the diatom *T. rotula* were host-specific in two independent and replicated experiments. The results of the host-specificity of the acceptor microbiomes on the diatom *T. rotula* demonstrate that algal-associated bacterial communities are more controlled by the algal host than by the initial inoculum composition of the bacterial source community. The suggestion that microalgae modulate the concentration and quality of their polysaccharides to attract specific heterotrophic bacteria is not new. Amin et al. (2012) showed that diatoms react to the presence of heterotrophic bacteria by selectively changing the quantity of polysaccharides in their exudates depending on the bacterial strain they were co-cultured with. However, whether the bacteria stimulate the algae to produce specific exudates or whether the algae produce exactly these polysaccharides to attract certain bacteria capable for B-vitamin biosynthesis for example, cannot be answered in this thesis. Nevertheless, the results of the microbiome assembly on *T. rotula* lend support to host factors more than the environmental bacterial diversity as dominant contributors in shaping the microbiome composition. Although the results do not reveal which factors are at play, this investigation revealed for the first time the host-specific microbiome assembly on the environmentally important diatom genus *Thalassiosira*.

Due to the demonstrated similarity of the newly established acceptor microbiomes on the diatom *T. rotula* which were inoculated with the diverse bacterial source communities of the donor cultures, the hypothesis was generated that the diatom *T. rotula* harbours a core microbiome. A core microbiome is typically defined to have a high degree of overlapping members in different microbiomes of the same species (Turnbaugh et al., 2009). Identifying the core species or OTUs is important to understand the ecology of interactions between microalgae and its bacterial microbiome because it has been proposed that these commonly occurring bacteria that appear in almost all microbiomes of the same host are likely critical for the function of the microbiome.

The co-culture experiments described in **Chapter 4 and 5** together revealed a core microbiome of 10 OTUs that had a high degree of overlap in the 25 newly assembled microbiomes on the *T. rotula* acceptor and contributed also most influential to the differences between the bacterial source communities and the acceptor microbiomes. Interestingly, these 10 OTUs also accounted for more than 80% to the total relative abundance of all OTUs in the acceptor microbiomes, possibly indicating the significance of these bacteria to the diatom *T. rotula* and / or the good adaptation of these bacteria to metabolise the exudates of *T. rotula* (**Chapter 5**). Consequently, the high abundance and occurrence of these 10 OTUs in the acceptor microbiomes suggested that these bacteria comprise the core microbiome of the diatom *T. rotula*. Therefore, the core microbiome of the diatom *T. rotula* consisted to large part of the bacterial genera *Alteromonas* and *Sulfitobacter*, which are often observed in association with microalgae (Schafer et al., 2002; Croft et al., 2005; Grossart et al., 2005; Sapp et al., 2007; Amin et al., 2015). Many studies have investigated the microbiome of specific marine microalgae (Ajani et al., 2018; Behringer et al., 2018; Majzoub et al., 2019), because microalgae-associated bacterial communities could be crucial to maintain the interactions between the microalgae and their environment. However, this is the first study that showed in repeated and replicated experiments that the environmentally relevant diatom *T. rotula* establishes a robust and reproducible bacterial core microbiome of 10 OTUs if it is offered a highly diverse and compositionally different bacterial source communities with up to 4406 OTUs. Additionally, the abundance of the 10 core bacterial OTUs in the microbiome of *T. rotula* evidences that these bacteria are likely crucial for the growth and performance of the diatom.

In summary, this thesis described the isolation of diatoms and bacteria from the environment and the development of an *in vitro* model system for reproducible laboratory studies to investigate the core microbiome of the isolated diatom species *T. rotula*. Identifying the core bacteria is essential to unraveling the ecology of host-microbe interactions, because it has been proposed that these commonly occurring bacteria that have a high overlap in all microbiomes with a particular species are likely critical to the function of that type of community. The description of the core microbiome of the diatom *T. rotula* takes the first step to better understand the principles that shape the microbiomes

on diatoms and a central step towards an understanding of the key bacteria associated with this ecological important diatom. The interactions between *T. rotula* and its stable and reproducible core bacterial members might be an interaction with global significance as *T. rotula* belongs to one of the most abundant genera of diatoms in the worldwide oceans (Malviya et al., 2016). It was shown **(Chapter 3)** that its growth is dependent on bacteria under vitamin scarce conditions in culture which are similar to the prevailing conditions in the ocean. Consequently, such interactions can have crucial effects on the marine food web and the global climate, because diatoms and marine microalgae in general are key primary producers (Field et al., 1998) responsible for more than 45% of the global net primary production, fixing billions of tons of inorganic carbon each year (Falkowski and Raven, 2007; Simon et al., 2009).

## 6.1 Outlook

The 10 OTUs that are described as the core microbiome of the diatom *T. rotula* in this thesis open new exciting possibilities for future research. A next step towards a deeper understanding of the function of these key bacteria associated with *T. rotula* could be the isolation of the 10 suggested core bacteria and the performance of co-culture experiments with each of these bacterial species on its own, in pairs and in multiple other combinations. Such experiments have the potential to reveal which of the 10 bacterial core OTUs are crucial for the growth and performance of the diatom *T. rotula* and which micronutrients are provided to the diatoms by the bacteria. Furthermore, it is now possible to perform co-culture experiments with the bacteria isolated in this thesis together with the axenic *T. rotula* acceptor culture. Such co-culture experiments have the potential to find out which bacteria are capable to provide important nutrients such as iron, vitamin $B_{12}$ or ammonium to the diatom *T. rotula* in return for DOC. The bacterial isolates might also allow to study the growth dependence of the 10 core bacterial OTUs to the secretion products of the diatom *T. rotula* and to elucidate the composition of the exudates of *T. rotula*. Moreover, it would be interesting to repeat the experiments described in **Chapter 5** and combine the used methods with metabolomics to unravel the factors which

shape the stable and reproducible microbiome of the diatom *T. rotula*. To sum up, the fact that the diatom *T. rotula* harbours a stable and reproducible core microbiome together with the availability of non-axenic and axenic *T. rotula* cultures represents an excellent model system to continue research with this ecological significant microalga.

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