# Balance of power:

# Dissolved nitrogen-to-phosphorus ratios and phytoplankton growth rate determine the balance between bottom-up and top-down processes in planktonic food webs

## DISSERTATION

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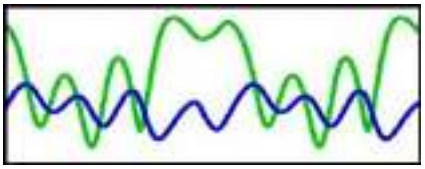

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**“ Ik wil de waarheid zoo dicht mogelijk benaderen en daarom alles abstraheeren tot ik kom tot het fundament der dingen. „**

- Piet Mondriaan, Painter, 1914 -

**“ Great attention gets paid to rainforests because of the diversity of life there. Diversity in the oceans is even greater. „**

- Sylvia Earle, Biological Oceanographer -

**“ As soon as you have entered into the pelagic wonderland, you will see that you cannot leave it. „**

- Johannes Müller to Ernst Haeckel, Physiologist, 1854 -

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# Summary

Phytoplankton are globally responsible for ~50% of the global oxygen production via primary production, fuelling food webs, and can alter biogeochemical cycles (Falkowski *et al.*, 1998; Field *et al.*, 1998; Falkowski & Raven, 2007). Grazing forms a massive loss factor of phytoplankton standing stocks. Since it can be challenging to measure variation in these relatively tiny organisms, most studies on planktonic predator-prey interactions overlook variation within groups and populations. It has recently become evident that neither prey populations nor predator populations can be viewed as homogeneous entities. Numerous physiological and behavioural differences can influence how predator-prey interactions act out in both phytoplankton and herbivorous zooplankton. Community organization may be influenced by variation in nutrient stoichiometry, cell quota, and nutritional requirements and, particularly intraspecific (within-population) variation. I therefore concentrated on nutritional stoichiometry as the changeable trait among populations since (i) the processes driving variation in this trait within populations are different in primary and secondary producers, which can result in mismatch phenomena, and (ii) the theories underlying how and why nutrient stoichiometry fluctuates are well developed, making accurate predictions feasible.

In the planktonic food web, a plethora of complex trophic interactions occur. I started this project with a series of grazing dilution experiments with seawater directly collected from the field during the most dynamic time period in temperate aquatic systems, the spring (phyto)plankton bloom. The aim of these grazing experiments was to investigate the role of zooplankton protists (<200 $\mu$m) in shaping bacterioplankton and phytoplankton bloom succession. We demonstrated that these zooplankton protists (i) actively select between and within phytoplankton and bacterioplankton prey populations, (ii) shift their grazing pressure depending on their nutritional requirements, as both prey items are plastic in their composition,

and (iii) play roles in termination of spring phytoplankton and bacterioplankton blooms. Notably, we were the first to highlight the role of zooplankton protists in shaping bacterioplankton bloom events.

The next step for me was to assess the role of an important bottom-up pressure in planktonic food webs: dissolved nutrient dynamics. I did this by extensively reviewing macronutrient compositions in growth media for phytoplankton. The *Food for thought* piece from this review highlighted the enormous diversity of media composition and the requirement for more unification. Phytoplankton mirror to some extent the resource availability, such as the natural fluctuation of light and nutrients within its elemental composition (i.e., food quality for herbivores). This variation in elemental composition is further influenced by growth rates, which culminates in most cases in the fact that fast growth is linked with a certain optimal nutrient content of the algae (less variation at a higher growth rate), whereas slow-growing algae can have a large array of different nutrient compositions (Hillebrand *et al.*, 2013). These patterns were identified between algal populations by averaging the individual responses of many different cells. The faster-growing populations furthermore approached Redfield-like values of nitrogen-to-phosphorus ratios of 16 (RR). As there are multiple ways to grow slowly for phytoplankton, but only one optimal, we advocate for a RR-correction of the N/P ratio in future use growth media.

Until now, it was unclear whether these patterns between algal populations also hold within single species and, even within populations, between single algal cells. To test this hypothesis, I conducted a laboratory experiment with an isogenic monoculture of the ubiquitous diatom *Phaeodactylum tricornutum*. With a gradient in growth rate obtained using 10 chemostats, I could determine the effect of growth rate on this diatom's selected traits, such as cellular stoichiometry and cell size, besides the hypothesised change in the coefficient of variation. Our results showed indeed changing stoichiometry as well as less intercellular variability in the faster-growing microalgal populations.

Lastly, I discussed the above findings in a broader context, including their potential repercussions for predator-prey interactions in planktonic food webs. It is suggested to proceed with this research with follow-up studies to ultimately improve our understanding of planktonic food web functioning and structuring, and their pivotal roles in biogeochemical cycling and fuelling the rest of aquatic food webs.

# Zusammenfassung

Phytoplankton ist weltweit für ~50 % der globalen Sauerstoffproduktion über die Primärproduktion verantwortlich, treibt Nahrungsnetze an und kann biogeochemische Kreisläufe verändern (Falkowski et al., 1998; Field et al., 1998; Falkowski & Raven, 2007). Die Beweidung ist ein massiver Verlustfaktor für den Bestand an Phytoplankton. Da es schwierig sein kann, die Unterschiede bei diesen relativ kleinen Organismen zu messen, werden in den meisten Studien über die Wechselwirkungen zwischen Räuber und Beute im Plankton die Unterschiede innerhalb von Gruppen und Populationen übersehen. In jüngster Zeit hat sich gezeigt, dass weder Beutepopulationen noch Räuberpopulationen als homogene Einheiten betrachtet werden können. Zahlreiche physiologische und verhaltensbedingte Unterschiede können die Art und Weise beeinflussen, wie sich Räuber-Beute-Wechselwirkungen sowohl im Phytoplankton als auch im pflanzenfressenden Zooplankton auswirken. Die Organisation der Gemeinschaft kann durch Variationen in der Nährstoffstöchiometrie, der Zellquote und dem Nährstoffbedarf und insbesondere durch intraspezifische (innerhalb von Population) Variationen beeinflusst werden. Ich habe mich daher auf die Nährstoffstöchiometrie als das veränderliche Merkmal zwischen den Populationen konzentriert, da (i) die Prozesse, die die Variation dieses Merkmals innerhalb der Populationen vorantreiben, bei Primär- und Sekundärproduzenten unterschiedlich sind, was zu Mismatch-Phänomenen führen kann, und (ii) die Theorien, die der Frage zugrunde liegen, wie und warum die Nährstoffstöchiometrie schwankt, gut entwickelt sind, so dass genaue Vorhersagen möglich sind.

Im planktonischen Nahrungsnetz gibt es eine Fülle komplexer trophischer Interaktionen. Ich begann dieses Projekt mit einer Reihe von Weideverdünnungsexperimenten mit Meerwasser, das während des dynamischsten Zeitraums in aquatischen Systemen der gemäßigten Zonen, der (Phyto-)Planktonblüte im Frühjahr, direkt aus dem Feld entnommen

wurde. Ziel dieser Weideexperimente war es, die Rolle von Zooplanktonprotisten (<200 µm) bei der Gestaltung der Abfolge von Bakterioplankton und Phytoplanktonblüte zu untersuchen. Wir konnten zeigen, dass diese Zooplankton-Protisten (i) aktiv zwischen und innerhalb von Phytoplankton- und Bakterioplankton-Beutepopulationen wählen, (ii) ihren Weidedruck je nach Nahrungsbedarf verändern, da beide Beutetiere in ihrer Zusammensetzung plastisch sind, und (iii) eine Rolle bei der Beendigung von Phytoplankton- und Bakterioplanktonblüten im Frühjahr spielen. Insbesondere waren wir die ersten, die die Rolle der Zooplanktonprotisten bei der Entstehung von Bakterioplanktonblüten hervorgehoben haben.

Der nächste Schritt bestand für mich darin, die Rolle eines wichtigen Bottom-up-Drucks in planktonischen Nahrungsnetzen zu bewerten: die Dynamik gelöster Nährstoffe. Zu diesem Zweck habe ich die Makronährstoffzusammensetzung in Wachstumsmedien für Phytoplankton eingehend untersucht. Die Denkanstöße, die sich aus dieser Untersuchung ergaben, verdeutlichten die enorme Vielfalt der Medienzusammensetzungen und die Notwendigkeit einer stärkeren Vereinheitlichung. Das Phytoplankton spiegelt in gewissem Maße die Verfügbarkeit von Ressourcen wider, z. B. die natürlichen Schwankungen von Licht und Nährstoffen in seiner elementaren Zusammensetzung (d. h. die Nahrungsqualität für Pflanzenfresser). Diese Schwankungen in der elementaren Zusammensetzung werden außerdem durch die Wachstumsraten beeinflusst, was in den meisten Fällen darin gipfelt, dass ein schnelles Wachstum mit einem bestimmten optimalen Nährstoffgehalt der Algen verbunden ist (weniger Schwankungen bei einer höheren Wachstumsrate), während langsam wachsende Algen eine große Bandbreite an unterschiedlichen Nährstoffzusammensetzungen aufweisen können (Hillebrand et al., 2013).

Diese Muster wurden zwischen den Algenpopulationen ermittelt, indem die individuellen Reaktionen vieler verschiedener Zellen gemittelt wurden. Die schneller wachsenden Populationen näherten sich außerdem Redfield-ähnlichen Werten des Stickstoff-

Phosphor-Verhältnisses von 16 (RR). Da es für Phytoplankton mehrere Möglichkeiten gibt, langsam zu wachsen, aber nur eine optimale, plädieren wir für eine RR-Korrektur des N/P-Verhältnisses in künftig verwendeten Wachstumsmedien.

Bislang war unklar, ob diese Muster zwischen Algenpopulationen auch innerhalb einzelner Arten und sogar innerhalb von Populationen zwischen einzelnen Algenzellen gelten. Um diese Hypothese zu testen, habe ich ein Laborexperiment mit einer isogenen Monokultur der ubiquitären Kieselalge *Phaeodactylum tricornutum* durchgeführt. Anhand eines Gradienten der Wachstumsrate, der mit Hilfe von 10 Chemostaten ermittelt wurde, konnte ich neben der angenommenen Veränderung des Variationskoeffizienten auch die Auswirkungen der Wachstumsrate auf ausgewählte Merkmale dieser Diatomee, wie z. B. die zelluläre Stöchiometrie und die Zellgröße, bestimmen. Unsere Ergebnisse zeigten in der Tat eine veränderte Stöchiometrie sowie eine geringere interzelluläre Variabilität in den schneller wachsenden Mikroalgenpopulationen.

Abschließend diskutierte ich die oben genannten Ergebnisse in einem breiteren Kontext, einschließlich ihrer möglichen Auswirkungen auf Räuber-Beute-Interaktionen in planktischen Nahrungsnetzen. Es ist notwendig, diese Forschung mit Folgestudien fortzusetzen um letztlich unser Verständnis der Funktionsweise und Strukturierung planktonischer Nahrungsnetze und ihrer zentralen Rolle im biogeochemischen Kreislauf und bei der Versorgung der übrigen aquatischen Nahrungsnetze zu verbessern.

# Samenvatting

Fytoplankton is wereldwijd verantwoordelijk voor ~50% van de mondiale zuurstofproductie via de primaire productie, voedt de voedselketen en kan de biogeochemische cycli veranderen (Falkowski et al., 1998; Field et al., 1998; Falkowski & Raven, 2007). Begrazing vormt een enorme verliesfactor van de fytoplanktonstand. Aangezien het een uitdaging kan zijn om de variatie in deze relatief kleine organismen te meten, gaan de meeste studies over planktonische predator-prooi interacties voorbij aan de variatie binnen groepen en populaties. Het is onlangs duidelijk geworden dat noch prooipopulaties noch roofdierpopulaties als homogene entiteiten kunnen worden beschouwd. Talrijke fysiologische en gedragsmatige verschillen kunnen van invloed zijn op de wijze waarop roofdier-prooi interacties zich voltrekken in zowel fytoplankton als herbivoor zooplankton. De organisatie van de gemeenschap kan worden beïnvloed door variatie in stoichiometrie van nutriënten, celquota en nutritionele behoeften en vooral door intraspecifieke (binnen-populaties) variatie. Ik heb me daarom geconcentreerd op de nutritionele stoichiometrie als de veranderlijke eigenschap tussen populaties, omdat (i) de processen die variatie in deze eigenschap binnen populaties veroorzaken verschillend zijn bij primaire en secundaire producenten, wat kan leiden tot mismatch-fenomenen, en (ii) de theorieën die ten grondslag liggen aan hoe en waarom nutriëntenstoichiometrie fluctueert goed ontwikkeld zijn, waardoor nauwkeurige voorspellingen haalbaar zijn.

In het planktonische voedselweb treden tal van complexe trofische interacties op. Ik ben dit project begonnen met een reeks begrazings- verdunningsexperimenten met zeewater dat rechtstreeks uit het veld werd verzameld tijdens de meest dynamische periode in gematigde aquatische systemen, de voorjaars(fyto)planktonbloei. Het doel van deze begrazings experimenten was de rol te onderzoeken van zoöplanktonprotisten (<200 µm) bij de vorming van bacterioplankton en fytoplanktonbloei. Wij toonden aan dat deze zoöplanktonprotisten (i)

actief selecteren tussen en binnen fytoplankton en bacterioplankton prooipopulaties, (ii) hun begrazingsdruk verschuiven afhankelijk van hun voedingsbehoeften, aangezien beide type prooien plastisch zijn in hun samenstelling, en (iii) een rol spelen bij de beëindiging van de voorjaarsbloei van fytoplankton en bacterioplankton. Wij waren met name de eersten die de rol van zoöplanktonprotisten bij de vorming van bacterioplanktonbloei benadrukten.

De volgende stap voor mij was het beoordelen van de rol van een belangrijke bottom-up druk in planktonische voedselwebben: de dynamiek van opgeloste voedingsstoffen. Ik deed dit door de samenstelling van macronutriënten in groeimedia voor fytoplankton uitgebreid te bestuderen. Het "Food for thought" stuk uit deze review benadrukte de enorme diversiteit in media samenstelling en de noodzaak voor meer unificatie. Aangezien fytoplankton tot op zekere hoogte de beschikbaarheid van hulpbronnen weerspiegelt, zoals de natuurlijke fluctuatie van licht en nutriënten in zijn elementaire samenstelling (d.w.z. voedselkwaliteit voor herbivoren). Deze variatie in elementaire samenstelling wordt verder beïnvloed door de groeisnelheid, wat er in de meeste gevallen toe leidt dat snelle groei gepaard gaat met een bepaald optimaal nutriëntengehalte van de algen (minder variatie bij een hogere groeisnelheid), terwijl langzaam groeiende algen een groot scala aan verschillende nutriëntsamenstellingen kunnen hebben (Hillebrand et al., 2013). Deze patronen werden geïdentificeerd tussen algenpopulaties door het gemiddelde te nemen van de individuele reacties van veel verschillende cellen. De sneller groeiende populaties benaderden bovendien Redfield-achtige waarden van stikstof/fosfor-verhoudingen van 16 (RR). Aangezien er voor fytoplankton meerdere manieren zijn om langzaam te groeien, maar slechts één optimale, pleiten wij voor een RR-correctie van de N/P-verhouding in toekomstige gebruikte groeimedia.

Tot nu toe was het onduidelijk of deze patronen tussen algenpopulaties ook gelden binnen afzonderlijke soorten en, zelfs binnen populaties, tussen afzonderlijke algencellen. Om deze hypothese te testen heb ik een laboratoriumexperiment uitgevoerd met een isogene

monocultuur van de veelvoorkomende diatomee *Phaeodactylum tricornutum*. Met een gradiënt in groeisnelheid, verkregen met 10 chemostaten, kon ik het effect bepalen van de groeisnelheid op de geselecteerde eigenschappen van deze diatomee, zoals cellulaire stoichiometrie en celgrootte, naast de veronderstelde verandering in de variatiecoëfficiënt. Onze resultaten toonden inderdaad een veranderende stoichiometrie en minder intercellulaire variabiliteit in de sneller groeiende microalgenpopulaties.

Ten slotte besprak ik bovenstaande bevindingen in een bredere context, met inbegrip van hun mogelijke gevolgen voor roofdier-prooi interacties in planktonische voedselwebben. Dit onderzoek moet worden voortgezet met vervolgstudies om uiteindelijk meer inzicht te krijgen in het functioneren en structureren van planktonische voedselwebben en hun centrale rol in de biogeochemische kringloop en het voeden van de rest van de aquatische voedselwebben.

# Chapter 1: General Introduction

Phytoplankton are globally responsible for ~50% of the global oxygen production via primary production, fuelling food webs, and can alter biogeochemical cycles (Falkowski *et al.*, 1998; Field *et al.*, 1998; Falkowski & Raven, 2007). Phytoplankton forms a group of aquatic drifters unable to swim against currents, which encompasses unicellular algae and cyanobacteria, i.e., autotrophic - photosynthetic to be precise - eukaryotes (algae) and prokaryotes (cyanobacteria). They produce organic matter via photosynthesis, converting gaseous carbon dioxide into oxygen and sugars using solar energy whilst assimilating dissolved nutrients. Their growth and overall functioning are impacted by ambient environmental conditions.

Here, the general introduction is divided into two parts, with the first part focusing on introducing planktonic food web functioning, and the second on the theoretical frameworks which I applied. More specifically, I discuss first how dissolved nutrients end up in aquatic systems, and how these nutrient loads and particularly load ratios can impact phytoplankton growth and phytoplankton community structure, whilst directly impacting trophodynamic interactions. Subsequently, I introduce the theoretical frameworks of Trait-based approaches and Ecological Stoichiometry (part II).

# Part I: Planktonic food web functioning

## 1.1: Theoretical background & Knowledge gaps

### 1.1.1: Nutrient cycles

*Natural nutrient loads*

Dissolved inorganic nutrients in aquatic systems fluctuate drastically during the year, due to abiotic and biotic processes. Nutrients are defined here as bioavailable salts in distinct structures containing elements that are essential to maintain biological processes in organisms. These elements end up in aquatic systems through physical and chemical processes, e.g., (i) erosion of weathered rocks with specific combinations of minerals, (ii) depositional injections of mineral particles from desert dust transported by storms (Goudie & Middleton, 2001), (iii) resuspension of the upper sediment layers by storms, (iv) river run-offs, and (v) certain anthropogenic activities (see below). These natural nutrient additions can vary in concentration once entering aquatic systems. This is due to abiotic processes such as evaporation and precipitation, biochemical fluxes such as uptake and ingestion, excretion and egestion, and remineralisation and sequestration of nutrients by microbes and protozoa via e.g., the microbial loop (see below; Azam *et al.*, 1983), the viral shunt (Wilhelm & Suttle, 1999) or the Biological Carbon Pump (BCP; Turner, 2015). Coastal areas are more impacted by these physical and chemical processes, as these areas are more affected by terrestrial processes compared to the open ocean.

Nutrient conditions in the open ocean are generally more stable than in coastal areas. In biogeochemical oceanography, there is the well-established Redfield ratio (Redfield, 1958). It represents the global elemental ratio of the macronutrients carbon (C), nitrogen (N) and phosphorous (P) of 106/16/1 in the seston - i.e., suspended organic (bioseston) and inorganic (abioseston) particulate particles in water bodies - collected from open seas, whilst resembling

the dissolved inorganic nutrient load ratios. Inorganic and sometimes organic nutrients are utilized by phytoplankton to be converted into biomass via photosynthetic processes. Whilst the elemental compositions of phytoplankton follow the natural dissolved nutrient load ratios to a certain extent (e.g., Redfield *et al.*, 1963; Sterner & Elser, 2002; Hillebrand *et al.*, 2013), the relevance and applicability of the Redfield ratio has been revisited numerous times (e.g., Tett *et al.*, 1985; Geider & La Roche, 2002; Klausmeier *et al.*, 2004a; Loladze & Elser, 2011; Hillebrand *et al.*, 2013). Fanning (1992) found that there are more oceanic nutrient regions with varying N/P ratios worldwide than earlier estimated, which was further discussed by Geider and La Roche (2002) and Martiny *et al.* (2013). In fresh water systems, the total nutrient loads are to a lower extent following the Redfield ratio, as these systems are more impacted by riverine inflows and sediment-water interactions. Despite this spatiotemporal variation globally, the Redfield ratio might be applied as a methodological approach to standardise how phytoplankton are kept in growth media with varying N/P supply ratios under controlled conditions in the laboratory to obtain a more unified and unambiguously control supply ratio.

### *Nutrient driven productivity cycles*

Dissolved inorganic nutrient concentrations fluctuate in aquatic systems seasonally. During winter, irradiance regimes in terms of photoperiods and light intensity and water temperatures drop in temperate regions, and even more in arctic ones. These less favourable growth conditions lead (among other loss factors) to lowered productivity (Sommer *et al.*, 1986; Sommer *et al.*, 2012). During these months, in temperate zones (where the field component of this dissertation took place, and hence the focus of the project), a pattern of increased mixing of the surface water layer – which introduces water originated from deeper nutrient-rich water layers during storm events – together with an increase of riverine inputs due to the reduced water temperature, and increased frequency of storm events during autumn and late winter/early spring can generally be observed. The combination of these conditions with reduced biological

activity of especially phytoplankton allows dissolved nutrients to redistribute into the surface waters where they had been depleted to some extent as a result of biological activity and subsequent sedimentation. Once it gets warmer and light intensity increases and photoperiods prolong during spring, the increasing water temperate, light availability and the accumulated nutrients allow microbial productivity to increase and spring plankton blooms, i.e., rapid formation of dominant surface growths of plankton populations, can occur. The enhanced microbial productivity stimulates phytoplankton growth, followed by an increase in zooplankton growth and subsequently by higher trophic levels. To sustain this growth, these blooms make use of the accumulated nutrients (e.g., Sverdrup, 1953; Loebl *et al.*, 2009), whilst affecting all corresponding trophic interactions in the system. Subsequently, during relatively warm summers, stratification of the surface layer occurs, which results in a period of non-renewal of nutrient-rich layers from below the surface layer, leading to a further decline in bioavailable nutrient loadings after the spring phytoplankton bloom event.

In late summer/early autumn, the release of nutrients from biological material can stimulate a second phytoplankton bloom event. More nutrients have redistributed in the system due to abiotic processes, such as the release of the summer stratification, and due to biotic processes, such as mineralisation and nutrient recycling, leading to more favourable growth conditions, which provides a small window for enhanced microbial and protozoan productivity. This window is well-known as autumn plankton blooms. Once nutrients are depleted and the grazing pressures on phytoplankton increased again, the bloom stops, reducing microbial productivity. During winter the plankton food web component of the ecosystem returns to its biological resting state, waiting for more favourable environmental conditions for growth to arrive again.

Since nutrient load cycles seasonally, phytoplankton growth is subject to nutrient-driven limitations. There are multiple ways to look at nutrient driven-limitations of plankton blooms. First, nutrient driven-limitations can be explained by Liebig's law of the minimum (Von Liebig,

1840), in which he stated that plant (and algal) growth is restricted by the limiting nutrient. When this nutrient is replenished, the next most limiting nutrient becomes the limiting one, and so on. Second, according to early resource competition theory, Liebig's law needs to be combined with the nutrient ratios as a key determinant of species composition (e.g., Tilman, 2020). And, as predicted by the nutrient-load hypothesis, not only the ratios but also the absolute nutrient loads play major role in species competition (Brauer *et al.*, 2012). When there are relatively high nutrient loads, there can be higher biomass build-ups, leading to an increasingly significant role of light limitation. Based on the community composition of the algae, this can lead to different competition outcomes in aquatic systems.

### 1.1.2: Phytoplankton dynamics in food webs

In the coastal and pelagic zones, primary production takes place by photoautotrophic eukaryotes and prokaryotes in mainly the sun-lit zone. This organic compound production is tightly linked to environmental conditions and to the phytoplankton community biodiversity, abundances and total biomass at a certain moment. Subsequently, this is linked to the community growth, which is the balance between reproduction (i.e., cell division) and mortality or removal. In plankton-focussed studies, we often calculate the net growth rate of an individual plankton species and plankton population and community by its exponential increase in cell number, Chlorophyll-*a* concentration or C biomass during a given length of time, assuming that biomass loss through mortality plays a minor role. The main factors responsible for the mortality component of these photoautotrophs which can be controlled in laboratory experiments are: (i) herbivory (Landry & Calbet, 2004), (ii) viral infections (Suttle, 2007), (iii) parasitism (Frenken *et al.*, 2017), (iv) sinking and sedimentation (Sakshaug *et al.*, 2009), and (v) physiological mortality of whole cells by both extremely unfavourable environmental conditions and natural ageing (senescence). In addition, environmental conditions need to be favourable to promote growth, i.e., cell division rate, as

otherwise most photoautotrophic cells will ultimately enter their specialized resting stages (cyst, spore) or enable programmed cell death based on the type of unfavourable condition(s) (Bidle, 2016; Belmonte & Rubino, 2019). Thus, environmental resources and conditions can determine the density, distribution, and structure of phytoplankton communities, and consequently of plankton communities as a whole (Tilman, 1982; Paerl *et al.*, 2014; Burson *et al.*, 2018).

Zooplankton grazing is a significant mortality factor of phytoplankton. An increasing number of studies show how microzooplankton can operate as a top-down regulator (e.g., Irigoien *et al.*, 2005; Löder *et al.*, 2011; Yang *et al.*, 2021). For example, microzooplankton (20-200 $\mu$m). They can consume ~60-75% of total primary producers (Landry & Calbet, 2004). Microzooplankton have different feeding modes or even express flexibility in their feeding modes (e.g., Stoecker *et al.*, 2017; Fig. 1.1). These shifts in feeding modes can be triggered by alterations in prey quality or predator-prey mismatches, e.g., change in the nutritional quality of their prey in terms of elemental stoichiometry (Sterner & Elser, 2002; Irigoien *et al.*, 2005), excretion of toxic substances by their prey (Irigoien *et al.*, 2005), or expression of less preferred traits such as size enlargements or spine and colony formation (e.g., Irigoien *et al.*, 2005; Litchman & Klausmeier, 2008).


**Figure 1.1: Microzooplankton feeding modes.** Three examples of common feeding modes among microzooplankton with (A) pallium-feeding, (B) engulfment by dinoflagellates, and (C) filter-feeding by ciliates.

A part of the microzooplankton community can also graze on bacterioplankton additionally to phytoplankton (Song *et al.*, 2009; Yang *et al.*, 2015). Overall, dinoflagellates are considered the major grazers of marine diatoms, because of their wide range of feeding strategies, whilst ciliates are recognized as major consumers of small phytoplankton species, flagellates and bacterioplankton (Bernard & Rassoulzadegan, 1990; Tillmann, 2004; Sherr & Sherr, 2007). As microzooplankton are a diverse group with different feeding modes and as they grow relatively fast, they can exert a strong grazing pressure on different prey (phytoplankton and bacterioplankton). Consequently, rapid successions in microzooplankton species that successively exert strong pressures on different prey groups, whilst releasing pressure on others, shape phytoplankton blooms (e.g., Löder *et al.*, 2011), and may also be able to shape bacterioplankton blooms.

Mesozooplankton ranging in size from 200 to 500 $\mu$m also exert a top-down pressure on phytoplankton, depending on food availability and quality, including resource availability, and on abiotic conditions like warming (e.g., Sherr & Sherr, 2002; Löder *et al.*, 2011; Boersma *et al.*, 2016). Mesozooplankton graze on both phytoplankton and microzooplankton. This in turn can impact the number and composition of smaller herbivores present.

The microbial loop is an important part of the planktonic food web which will be revisited below (Azam *et al.*, 1983). This loop is mediated by bacterioplankton and protozoans and refers to the regeneration of nutrients and their return to the lower trophic levels of the food chain. Bacterioplankton and protozoans utilize dissolved and particulate organic carbon and certain metabolic by-products produced mostly higher up in the food chain by for example sloppy feeding by consumers, excretion, exudation or organismal degradation (Jumars *et al.*, 1989).

# 1.2: Global and regional threats

*Global change*

We are experiencing a period of climate change at a pace that is unprecedented in geological history. This climate change is impacting biospheres by warming, elevated $pCO_2$, ocean acidification, and solar irradiance levels, as discussed in the Intergovernmental Panel on Climate Change's fifth assessment report (IPCC; Pachauri *et al.*, 2014) and by Duarte (2014). This change also affects marine and freshwater plankton communities, including phytoplankton. In the review by Beardall *et al.* (2009), they give the status quo on the impacts of global climate change on the physiological properties, productivity, and assemblage composition of marine phytoplankton. For instance, warming can lead to altered metabolic rates and therefore growth rates (Raven & Geider, 1988), selective grazing pressure (e.g., Kleppel, 1993; Boersma *et al.*, 2016), apart from stimulated stratification of the water column, which in low and mid-latitudes will lead to nutrient limitations in the surface layer that can impact species-specific phytoplanktonic traits (Raven & Geider, 1988) and communities in species composition and diversity (Burson *et al.*, 2016).

Anthropogenic activities are another factor that has led to changes in nutrient loads ending up in rivers running towards nearshore coastal and marine environments and lakes (e.g., Loebl *et al.*, 2009; Grizzetti *et al.*, 2012). The anthropogenically induced nutrient over-enrichment is known as eutrophication, which leads to higher nutrient loads of spatially-specific nutrients, shifting nutrient load ratios, and hence altering phytoplankton (and bacterioplankton) growth rates, thereby effecting the overall nutrient cycling and zooplankton standing stocks in planktonic food webs. Moreover, when looking at this eutrophication in combination with the forecasted future of the nutrients nitrogen (N) and phosphorus (P), it is getting even more crucial that we increase our reduction measures.

Eutrophication of N and P comes from different sources. For N, the main source is fertilizers and atmospheric N-deposition resulting from fertilizer usage. N-enrichment gets anthropogenically enhanced next by terrestrial runoff of agricultural waste products, e.g., by rising atmospheric inputs and $N_2$ fixation (Fanning, 1992). For P, the global sources, such as phosphate rock reserves is finite and declining with only enough for 50-200 years if our current usage rate continues (Herrera-Estrella & López-Arredondo, 2016). As P is getting scarcer in the future, N/P ratios will inevitably increase in water masses worldwide (e.g., Grizzetti *et al.*, 2012), albeit from different spatial *in-situ* ratios (Martiny *et al.*, 2013).

Legal reduction measures installed to minimize the marine eutrophication to date, such as caused by the usage of N+P-rich fertilizers especially the addition of urea and ammonium in agriculture and P-rich detergents in households, are also leading to altered *in-situ* elemental ratios (e.g., Riegman, 1995; Turner *et al.*, 2003; Grizzetti *et al.*, 2012; Burson *et al.*, 2016). Examples of such reduction measures are for chemical P in fertilizers: (i) developing crop varieties with higher P utilization efficiency, (ii) designing alternatives with distinct chemical structures, and (iii) producing systems, such as the usage of microbial inoculants to transfer immobilized P from the soil (Herrera-Estrella & López-Arredondo, 2016). For N, examples of reduction measures are (i) lowering the amount of diffuse pollution produced by agriculture and (ii) minimizing the application of mineral fertilizers (Wendland *et al.*, 2005).

Oligotrophication measures could lead to unwanted side effects. For example, as in Lake Constance in Southern Germany, where the fish population has declined as a side-effect of these mitigation measures (Gaedke & Schweizer, 1993; Sommer *et al.*, 1993). The reduced P loads led to a declined phytoplankton biomass in summer, whilst in spring the biomass stayed constant. This reduced summer biomass has likely cascaded up via zooplankton to the fish level. Another example of unbalanced reduction loads is an offshore N/P gradient in the North Sea, due to a less effective decline of N-rich sources (Burson *et al.*, 2016). Therefore, it is of imperative importance that the intensity of these reduction measures needs to be profoundly

planned, discussed, and biomonitored at multiple levels after implementation to mitigate unwanted side effects. The findings of this thesis contribute to our understanding of planktonic food web functioning, and therefore aiding to more profound analyses of the obtained biomonitoring data sets. A promising approach to further study how changes in environmental conditions influence plankton, is using different ecological frameworks, such as Trait-based ecology (TBE) and Ecological Stoichiometry (ES).

# Part II: The applied theoretical frameworks

## 1.3: Trait-based ecology & Ecological Stoichiometry

### 1.3.1: Trait-based ecology

Trait-based ecology provides a way to compare different organisms and functional groups with varying spatiotemporal characteristics. It is a swiftly advancing branch of ecology in which population and community ecology are combined with evolutionary ecology (Kiørboe *et al.*, 2018). The major benefit of using trait-based approaches, is that it advances the search for general principles in community ecology by focusing on individuals characterized by traits rather than species present in an ecosystem (Kiørboe *et al.*, 2018). *Traits* are used to describe characteristics of organisms (here planktonic cells) and are defined here as stated in Violle *et al.* (2007): "Any morphological, physiological or phenological feature measurable at the individual level, from the cell to the whole-organism level, without reference to the environment or any other level of organization". Functional traits refer to specific traits that impact organismal or cellular fitness indirectly by affecting growth, reproduction, and/or survival (Violle *et al.*, 2007). In this branch of ecology, a framework is built on the following four themes: traits, environmental gradients, the interaction milieu, and performance currencies (Mcgill *et al.*, 2006). Traits are powerful tools to quantify the functional biogeography of a system or cell/organism, the diversity of a system, and/or its potential resilience to perturbations (Martini *et al.*, 2021).

By continuously assessing the usage of potential traits among ecosystems, the traits typology for plankton by Litchman and Klausmeier (2008), is increasingly expanding (Martini *et al.*, 2021; and references therein). Within these typologies of aquatic functional traits, traits are classified based on their trait type: Morphological, Life history, Physiology and/or

Behavioural, and on ecological function: Resource acquisition, Growth, Reproduction and/or Survival.

Margalef (1978) was one of the first who explored trait-based approaches with his seminal so-called mandala. By plotting the responses of these different functional groups along gradients of turbulence against nutrients, he could predict the environmental occurrence of different functional phytoplankton groups within the nutrient-turbulence space.

Earlier work in the development of trait-based approaches within the aquatic sciences was performed by Sommer (1984). By performing a series of nutrient competition experiments in chemostats with natural phytoplankton community assemblages under steady-state conditions with either a continuous or pulsed P supply along a dissolved silicon (Si)/P supply, he proposed there are three major nutrient acquisition strategies: velocity-adapted, storage-adapted, and affinity-adapted phytoplankton species. Velocity-adapted species are characterized by high maximum nutrient uptake rates ($\upsilon_{max}$) and high maximum growth rates ($\mu_{max}$), and hence benefit from nutrient pulses to grow fast, which leads to oscillating species numbers in the plankton community. Both storage-adapted species with high $\upsilon_{max}$ but lower $\mu_{max}$, and affinity-adapted species that have low half-saturation constants for nutrient uptake ($K$), that are advantageous under nutrient limitation, respond overall slower to such nutrient pulses, hence stabilizing the phytoplanktonic community composition. This partly explains the huge shifts in species dominance in the community assemblages over the course of a temperate phytoplankton bloom event, seen from a bottom-up approach. Moreover, by assessing the experimental results on species number and the Shannon-Wiener diversity index (H'; Weaver & Shannon, 1963), he found that a nutrient-pulsed supply led to a higher species number and diversity along the Si/P gradient. Since organisms and cells can have distinct trait values (termed attributes; Violle *et al.*, 2007) over various environmental gradients, trait-based

ecology is a good tool to study inter- and intraspecific variability, and to assess the applicability of certain traits to study trait flexibility (Martini *et al.*, 2021).

Mechanisms generating trait flexibility in organisms and cells leading to flexibility in trophic interactions and nutrient uptakes, and hence ultimately to community composition can be classified into the following three major categories: phenotypic plasticity, rapid evolution, and species sorting. These mechanisms differ in the extent of flexibility they provide and the timescale on which they operate.

Phenotypic plasticity for example, supports numerous behavioural, morphological, and physiological trait alterations on an individual level, which are expressed in response to an environmental cue. Notably, such trait changes do not involve changes to the genetic material, and can therefore be intra-generational and reversible. For example, when phytoplankton are threatened, phytoplanktonic cell enlargement could aid predator avoidance. Also, phytoplankton mucilage production forms a physical barrier to protect against a fungal parasite attack, grazer deterrence and modified buoyant properties and hence predator-prey encounter rates (Reynolds, 2007; Litchman & Klausmeier, 2008; Van Der Stap *et al.*, 2008; Van Den Wyngaert *et al.*, 2022).

The other two mechanisms, rapid evolution and species sorting on the other hand occur over the course of multiple generations. Together, these mechanisms contribute to the wide variety of traits in organisms and cells. This leads to flexibility in trophic interactions and nutrient up-takes, and hence ultimately to functional community structure.

## *Phytoplankton in trait-based approaches*

Phytoplankton and their corresponding loss factors play key roles in the health state and resilience of aquatic ecosystems and their functioning. In particular, as elements can travel up

the food chain (Boersma *et al.*, 2008; Malzahn *et al.*, 2010). It is becoming increasingly important to unravel how trophic interactions in planktonic food webs are affected by short-term pulse and long-term press alteration in environmental conditions caused by global climate change and anthropogenic actions (Donohue *et al.*, 2016).

With a trait-based approach more communities from numerous systems can be compared to better assess their ecological functioning. For example, it was found that the presence of the N-fixation trait in phytoplankton communities, such as diazotrophy in cyanobacteria forms an indicator for N-poor systems (Litchman *et al.*, 2010). A comparable example of an applied approach in different fields of ecology is the emerging application of the seascape-approach (Boström *et al.*, 2011). This approach focusses on the interconnectivity of several habitats, whilst including several landscape ecological concepts and theories to coastal environments in the fields of conservation and restoration ecology. The same view as with the seascape-based approach can be applied to the complex interplay between functional groups in planktonic food webs to improve biomonitoring efforts, particularly when combined with trait-based approaches (e.g., Godhe *et al.*, 2013; Godhe *et al.*, 2016; Martini *et al.*, 2021).

### 1.3.2: Ecological stoichiometry

One of the key traits of organisms is their elemental composition, which is influenced by nutrient concentrations. The importance of elemental composition in elemental quota and ratios is described and discussed within the framework of Ecological stoichiometry (Sterner & Elser, 2002). This framework focuses on the balance of and linkages between numerous chemical elements and energy (C) flows in ecological systems and interactions (Meunier *et al.*, 2017; Welti *et al.*, 2017). One of these linkages are predator-prey interactions in planktonic food webs (Meunier *et al.*, 2012b; Meunier *et al.*, 2018). The elemental composition of the prey (food quality) has implications for its consumer(s), i.e., the

food quality for a consumer depends on the food composition matching the needs in terms of elemental ratios of the consumer. Food quality determines the consumer's development, survival and reproduction. Strong nutrient limitations lead to low food quality resources which can impact the consumer's growth and selective feeding (Hantzsche & Boersma, 2010; Meunier *et al.*, 2016).

## *Correlation of algal elemental composition with growth rate*

The early field of algal growth kinetics focuses on the relation of algal growth to nutrient supply. Droop (1983) demonstrated over the years with more than 30 empirical experiments that phytoplankton growth is limited by nutrient availability, such as ammonium, iron, N, P and Si, and that the intracellular nutrient quota is more linked to growth rate than extracellular nutrient loads. This chemostat work on phytoplankton growth kinetics continued, showing lower homeostasis with slower-growing - by lowered chemostat dilution rates, and hence nutrient supplies - algal growth rates (Rhee, 1978; Goldman *et al.*, 1979) and cyanobacterial growth rates (Mouginot *et al.*, 2015; Garcia *et al.*, 2016). This was reviewed in a meta-analysis by Hillebrand *et al.* (2013), in which they analysed 43 data sets of differently growing phytoplankton cells under distinct limitations with a whole-taxa approach in both mono- and polycultures. This analysis revealed that when the cellular N/P ratio is more constrained, cells grow faster, approaching the Redfield ratio of N/P=16 (**Fig. 1.2**). Also, the faster-growing phytoplankton populations likely have a lower intercellular trait variation in their elemental composition.

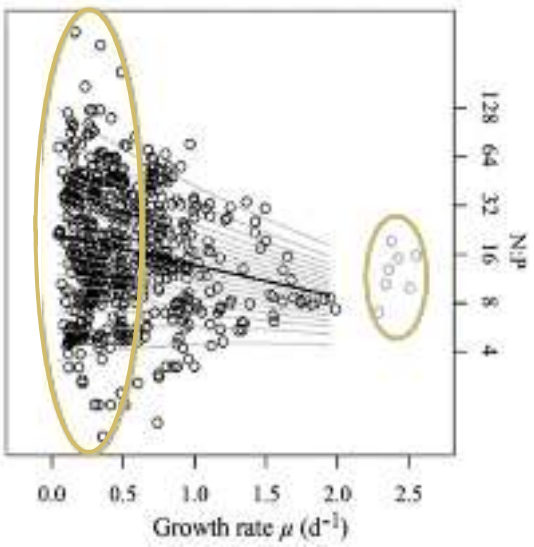


**Figure 1.2: The relation between nutrient supply ratio and phytoplankton growth rate.** Negative correlation between variability in molar N/P ratio and growth rate indicated by the length of the gold-coloured circles in phytoplankton with a whole-taxa approach (After Hillebrand *et al.*, 2013).

Despite the well-established field of algal growth kinetics and nutritional quality, there are still multiple open fundamental questions (Moreno & Martiny, 2018; Danger *et al.*, 2022). One of them is whether the trends described above would also hold within a differently growing single (monogenetic) algal population. If so, these trends would be linked to phenotypic plasticity and trait flexibility in phytoplankton cells, since faster-growing phytoplankton populations have more constrained elemental compositions (Hillebrand *et al.*, 2013). Notably, phytoplankton have less homeostasis and more elemental plasticity compared to higher trophic levels (Van De Waal & Boersma, 2012). This would contribute to disentangling the enigmatic and long-standing conundrum, the paradox of the plankton (Hutchinson, 1961; Record *et al.*, 2014). When phytoplankton grow slowly under higher nutrient limitations, they could have a higher inter- and intraspecific and intercellular trait variability, as there are more ways of cells to grow slowly than fast, i.e., there are a myriad of ways cells could be limited in their growth. This increased trait variability with lower growth rates creates a wider prey quality (e.g.,

morphological and biochemical composition) window (broader buffet) for zooplankton to select from to reach their own nutritional demands. This could have repercussions for planktonic food webs, such as a decrease in predator fitness and predator diversity. Also, as elements can cascade up in the planktonic food web, the trophic transfer can impact for example fish and lobsters (Boersma *et al.*, 2008; Malzahn & Boersma, 2009; Schoo *et al.*, 2012). Functional diversity in plankton communities is positively correlated with community stability, productivity, resource use efficiency, and resistance to environmental pulse disturbances, such as storm and heatwave events, albeit negatively to resilience (Ptacnik *et al.*, 2008; Baert *et al.*, 2016; Vallina *et al.*, 2017).

## 1.4: Dissertation outline

Within this dissertation, I combined the ecological frameworks of trait-based ecology and ecological stoichiometry, whilst including the reasoning of the novel vertical upscaling framework to tackle our research objectives on the bottom-up and top-down pressures which phytoplankton experience in planktonic food webs (Meunier *et al.*, 2017; Van Velzen et al., In prep.). Using these approaches, we aim to unravel whether a wider window of available prey diversity can sustain a higher diversity of grazers within the planktonic food web. Furthermore, we propose that this can address an interplay of fundamental ecological concepts and theories, such as the paradox of the plankton (Hutchinson, 1961). This project is part of the 2$^{nd}$ consortium of the DFG's (German Research Foundation) priority programme DynaTrait to assess the impacts of dynamic traits in aquatic predator-prey interactions from an individual perspective scaling up to ecosystem level in both empirical and theoretical ecological research studies.

In bloom succession of planktonic food webs, predator-prey interactions are well-studied to date (Sommer *et al.*, 1986; Sommer *et al.*, 2012). These complex interactions can

change by both pressure from above (top-down control) and below (bottom-up control). Examples of top-down controls are grazing (Sommer *et al.*, 1986; Löder *et al.*, 2011), viral lyses (e.g., Suttle, 2007; Brussaard *et al.*, 2008; and references therein) and parasitic attacks (Frenken *et al.*, 2017). Examples of bottom-up controls are the bioavailability of dissolved nutrients, sinking (Sakshaug *et al.*, 2009), and abiotic parameters, such as the light regime and ambient temperature. Ultimately, such controls could lead to nutritional mismatches in trophic interactions in planktonic food webs (Malzahn *et al.*, 2007; Boersma *et al.*, 2016), as aforementioned. The focus within my dissertation project was on dissolved nutrient availability as the main bottom-up control and grazing by micro and mesozooplankton (~200-500 $\mu$m) as the main top-down control.

As a first approach to tackle the project's main objective, the focus was on the trophic interactions from a top-down perspective occurring during a highly dynamic temporal period in the planktonic food web, the spring bloom. The top-down pressures by herbivores from the size fraction of microzooplankton (20-200 $\mu$m) exerted on microplankton are receiving increasing attention over the last decades (Irigoien *et al.*, 2005; Löder *et al.*, 2011). It is known that several microzooplankton genera can feed selectively or alter their feeding behaviour based on the available prey quality and their own nutritional demands (e.g., Meunier *et al.*, 2012a; Meunier *et al.*, 2012b) leading to a higher diversity in feeding modes and hence flexibility in top-down regulation by microzooplankton.

Microzooplankton forms a loss factor for both phytoplankton and bacterioplankton, leading to less microbial activity in terms of primary production by removing nutrients from the systems, and remineralisation by resupplying nutrients. Using the unified approaches as aforementioned, we could address the following key knowledge gaps hitherto within these aquatic trophic interactions: (i) Shifts in prey selection between and within phytoplanktonic and bacterioplanktonic functional groups by nano- and microzooplankton over the bloom

succession; (ii) the top-down pressure exerted by nano- and microzooplankton on bacterioplankton in terms of driving bacterioplankton succession; and (iii) whether nano- and microzooplankton can terminate both phytoplankton and bacterioplankton blooms. Moreover, we studied this for the first time in a high resolution over the succession of a temperate spring plankton bloom event. We aimed to sample at least five times during the plankton bloom succession to include the five main theoretical distinct phases (illustrated with the dashed lines in **Fig. 1.3**). The results of this study on grazing by nano- and microzooplankton in the perspective of planktonic food web functioning are included in **Chapter 2**, provide room for synthesis in the field of predator-prey interactions in other biospheres, and are further discussed in **Chapter 5**.

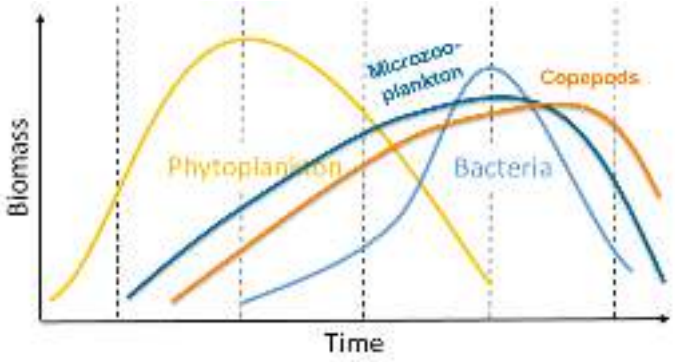


**Figure 1.3: Temperate plankton bloom dynamics.** Schematic overview of the target sampling moments of the five distinct theoretical phases of a simplified temperate bloom event.

To evaluate the role of zooplankton protists in shaping phytoplankton, and bacterioplankton blooms (**Chapter 2**), a field study was performed at the Helgoland Roads in the German part of the North Sea (54º 11.3'N, 7º 54.0'E). Here, the Helgoland Roads long-term time series is stationed (Wiltshire *et al.*, 2008; PANGAEA, 2004; http://www.pangaea.de). This marine roads is located between the islands Helgoland and Düne and ~60 km off the estuaries of rivers Elbe and Weser (Franke *et al.*, 2004). The phytoplankton time series collection started in 1962, and the (meso)zooplankton one in 1974 on a work-daily basis (Greve *et al.*, 2004; Wiltshire & Manly, 2004). Furthermore, tackling these objectives provides ultimately novel

insights into the roles that microzooplankton have in complex trophic interactions in aquatic food webs relevant for both empirical and theoretical plankton researchers, and room for synthesis in the field of predator-prey interactions in other biospheres.

There is a high diversity in the proportions in which macronutrients are added to growth media for phytoplankton. Globally, water masses and bodies contain high variability in dissolved macronutrient ratios, which led to a myriad of distinct growth media compositions. **Chapter 3** is dedicated to how we might standardize our media composition for laboratory studies to obtain studies with similar experimental controls which are more straightforward to compare. Additionally, further recommendations are provided to optimise future media usage.

To further unravel the complex trophic interactions happening in planktonic food webs, the system was simplified to the fundamentals of how a single type of algal prey is affected by different nutrient loads in its own functional traits. The focus was redirected on how distinct nutrient loads impact algal prey growth leading to altered phenotypic plasticity, trait variations, and food qualities. In **Chapter 4**, we determine whether the hypothesized trends in combined trait variation with N/P ratio along a growth rate gradient within the meta-analysis by Hillebrand *et al.* (2013) also hold within a single genotype of a model phytoplankter.

Last, in **Chapter 5** the findings of my dissertation will be presented, discussed and scrutinized in the broader context of plankton ecology and changing plankton food web structure and functioning. I'll furthermore provide an outlook on future research opportunities.

## 1.5: Hypotheses dissertation

**Hypothesis I:** I hypothesize that based on the diverse feeding modes of zooplankton protists, these consumers can select for specific prey types based on their nutritional requirements, thereby exerting important grazing pressures on parts of the bacterioplankton and phytoplankton standing stocks. I test this hypothesis with a series of grazing dilution experiments in **Chapter 2**.

**Hypothesis II:** Faster-growing phytoplankton will approach the Redfield ratio and hence contain more P (in a P-limited system) forming a higher quality food source to consumers, ultimately leading to higher consumer fitness. The expectation is that grazers would select for the faster-growing phytoplankton cells, and hence a higher quality food source. We checked whether this holds true in the plankton food web by performing both a series of grazer dilution experiments with sea water collected from the field (**Chapter 2**) and two laboratory experiments using a single microzooplankton species (**Chapter 5**).

**Hypothesis III:** Faster-growing phytoplankton populations have a lower intercellular trait variation (**Chapter 4**) under optimal growth conditions, because they are growing more optimal under nutrient conditions close to Redfield Ratio. These optimal conditions might be applied to the way phytoplankton are kept in the laboratory, by modifying the macronutrient supply ratios (**Chapter 3**).

**Hypothesis IV:** Slower-growing phytoplankton populations will create a broader window of prey qualities and types, leading to a higher diversity and species richness in consumer species maintained in a plankton food web. This hypothesis is tested in **Chapter 5** combined with a synthesis using the data from **Chapter 2** in **Chapter 5**.

# 1.6: Publications & Manuscripts

***The thesis is based on the following papers:***

**Paper I** (Chapter 2) — **Klip HCL**, Suarez-Caballero JL, Boersma M, Fuchs BM, Wiltshire KH, Meunier CL. Rise and fall: Nano- and microzooplankton grazing pressure drive species succession and bloom termination of their phyto- and bacterioplankton prey. *Manuscript.*

**Paper II** (Chapter 3) — **Klip HCL**, Meunier CL, Boersma M. Thoughts for food: Why are macronutrient ratios of many growth media for phytoplankton so different from the Redfield ratio? *Under review in Journal of Plankton Research.*

**Paper III** (Chapter 4) — **Klip HCL**, Meunier CL, Boersma M. Less variability when growing faster? Experimental assessment of the relationship of growth rate with functional traits of the marine diatom *Phaeodactylum tricornutum*. *Under review in Hydrobiologia.*

***My contribution to the papers:***

**Paper I**: The scientific concept was developed by me together with MB, BMF and CLM. JLSC and I collected the samples and conducted the series of dilution experiments. JLSC processed the samples for phytoplankton and zooplankton protists, and BMF for bacterioplankton. I, JLSC and CLM analysed the data. I and CLM managed the project. I wrote the manuscript, which was commented on and edited by the co-authors.

**Paper II**: The scientific concept was developed by me together with MB and CLM. I performed the literature review and synthesized the two supporting tables. I wrote the manuscript, which was commented on and refined by the co-authors.

**Paper III**: I developed the scientific concepts with the help of CLM and MB. I conducted the experiments, analysed the samples, and evaluated the data. I wrote the manuscript, which was commented on and edited by the co-authors.

# Chapter 2: Role of microzooplankton in bloom events

## Rise and fall: Nano- and microzooplankton grazing pressures drive succession and bloom termination of their phyto- and bacterioplankton prey

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# Abstract

Nanozooplankton and microzooplankton are major functional groups in microbial food webs which can exert an important grazing pressure on phytoplankton and bacterioplankton. These diverse groups comprise species which have different feeding modes, and which can feed selectively depending on their dietary needs. However, little is known on the seasonal dynamics of the grazing pressure exerted by nano- and microzooplankton on different prey taxa and traits, and on the extent to which nano- and microzooplankton may drive species succession and bloom termination of both phytoplankton and bacterioplankton. Here, we conducted a series of grazing dilution experiments with natural marine communities over the course of a temperate spring bloom event, and combined those with in-situ field measurements of relevant abiotic parameters to determine the combined role of nano- and microzooplankton in driving the abundance and taxonomic composition of their prey. Our data show that nano- and microzooplankton can shift their grazing pressure (i) between phytoplankton and bacterioplankton, as well as (ii) within these groups, which (iii) shapes the composition of phyto- and bacterioplankton blooms and substantially contribute to the termination of these blooms. This study highlights the considerable extent to which grazing by nano- and microzooplankton is driving the succession and bloom termination of their bacterioplankton and phytoplankton prey, and overall strengthens our understanding of microbial food web functioning during spring blooms.

# Introduction

Nano- and microzooplankton (defined here as heterotrophic protists between 2-20 $\mu$m and 20-200 $\mu$m, respectively) play key roles in aquatic food webs, and exert a substantial top-down pressure on both bacterioplankton and phytoplankton (e.g., Šimek *et al.*, 1999; Irigoien *et al.*, 2005; Löder *et al.*, 2011; Schmoker *et al.*, 2013; Yang *et al.*, 2015). As such, those groups (i) link small primary producers and bacterioplankton to higher trophic levels, (ii) compete with mesozooplankton for the same resources, and (iii) contribute substantially to the rapid recycling of nutrients (Irigoien *et al.*, 2005; Fenchel, 2008). Moreover, nano- and microzooplankton affect the energy pathways within the microbial loop (Azam *et al.*, 1983; Fenchel, 2008) by feeding selectively on certain prey groups (e.g., Löder *et al.*, 2011) and on organisms of specific nutritional value (Meunier *et al.*, 2012b; Meunier *et al.*, 2018), impacting the carbon flow in planktonic food webs (Pasulka *et al.*, 2015; Talmy *et al.*, 2019). Despite their ecological importance, detailed studies on the influence of nano- and microzooplankton grazing on the seasonal dynamics of their phytoplankton and bacterioplankton prey are rare.

Zooplankton protists comprise species which have different feeding modes and strategies, enabling them to efficiently consume and select for different types of prey regarding prey taxa (Stoecker *et al.*, 1981; Stoecker *et al.*, 1986; Buskey, 1997), or sizes (Andersson *et al.*, 1986; Chrzanowski & Šimek, 1990; Jakobsen & Hansen, 1997). For instance, for dinoflagellates, direct engulfers specialize on small prey, i.e., bacterioplankton and (pico)phytoplankton, and pallium feeders specialize on large (micro)phytoplankton (García-Oliva & Wirtz, 2022). Within ciliates, suspension and deposit feeders preferentially consume small prey such as bacterioplankton and nanophytoplankton. Physical properties of prey such as size, its elemental and biochemical composition are also an important factor determining its suitability as food for grazers. For instance, prey quality can be defined in terms of elemental

ratios (Sterner & Elser, 2002), a prey trait which has also been shown to drive microzooplankton feeding behaviour (Meunier *et al.*, 2012a; Meunier *et al.*, 2012b). Consequently, the diversity of feeding behaviour which zooplankton protists can exhibit, combined with their high grazing potential, may enable them to have a significant impact on the structure and dynamics of a wide variety of prey. During bloom events for instance, nano- and microzooplankton can respond quickly to increasing prey availability (Johansson *et al.*, 2004; Aberle *et al.*, 2007), and it has been proposed that the algal species that manage to form a bloom, are in fact those able to escape predation pressure by zooplankton protists (Irigoien *et al.*, 2005; Löder *et al.*, 2011). However, there is still little evidence for the role microzooplankton play in structuring the blooms, not only of specific phytoplankton species, but also of specific bacterioplankton clades, and in terminating these blooms.

In temperate systems, phytoplankton seasonal dynamics are characterized by two blooms, the first and most intense one occurring in spring and the second one in summer (Wiltshire *et al.*, 2008). Generally, the onset of phytoplankton bloom dynamics in marine environments are described from a bottom-up perspective, with phytoplankton abundances increasing when nutrient and light conditions are sufficient for positive growth, and then declining because of nutrient limitation (e.g., Smetacek, 1999), followed by top-down pressures exerted by organisms ranging from viruses to mesozooplankton that may shape these blooms (e.g., Fuhrman, 1999; Irigoien *et al.*, 2005; Löder *et al.*, 2011). In particular, Irigoien *et al.* (2005) pointed out that defence mechanisms of phytoplankton cells (e.g., large cell sizes, colonies or spine formation) combined with selective predation of zooplankton protists open a loophole enabling less edible, unfavoured phytoplankton species to form blooms. As knowledge on the cumulative influence of those factors on phytoplankton bloom assemblages and dynamics is still limited (Brussaard *et al.*, 1995; Aberle *et al.*, 2007) studies on microzooplankton grazing under natural conditions are imperative.

The rapid increase in phytoplankton biomass characterizing spring blooms is closely followed by an increase in bacterioplankton abundances which often reprocess about half of the net primary production in the microbial loop (Azam, 1998). The dynamics of bacterioplankton during marine algal spring blooms are considered to be primarily driven by bottom-up processes, and especially by the quantity and quality of phytoplankton-derived organic matter (Azam, 1998; Riemann *et al.*, 2000; Fandino *et al.*, 2001; Teeling *et al.*, 2012). For instance, Teeling *et al.* (2012) linked specific carbohydrate-active enzymes and phosphate acquisition strategies of different bacterioplankton clades that prosper at distinct stages of the spring bloom to phytoplankton-derived substrate availability, and suggested that substrate quality provide ecological niches in which specialized bacterioplankton populations can bloom. Interestingly, limnological studies have long recognized that selective grazing by zooplankton protists can also shape bacterioplankton communities (e.g., Šimek *et al.*, 1999), but this knowledge has not really been incorporated in marine ecology. Indeed, microzooplankton selectively consume specific bacterioplankton prey depending on their size, motility, or physicochemical surface characteristics (Jürgens & Matz, 2002) which can structure bacterial communities toward grazing-resistant cells (Šimek *et al.*, 1999). We will investigate the potential role that grazing plays in shaping marine bacterioplankton communities during spring blooms, influencing the identity of the blooming populations, and driving the termination of these blooms.

Here, we focussed on the top-down control of nanozooplankton and microzooplankton during a spring bloom event in a temperate shelf sea system. The main aim of our study was to assess the role of zooplankton protists in driving species succession of their phytoplankton and bacterioplankton prey, and in terminating an algal bloom event. We conducted a series of dilution experiments during the course of a spring bloom to (i) establish and measure the feeding preference of zooplankton protists between and within phytoplankton and bacterioplankton, (ii) quantify the top-down pressure exerted by zooplankton protists on

phytoplankton and on bacterioplankton, and (iii) evaluate the role of zooplankton protists in shaping phytoplankton and bacterioplankton communities.

# Material and methods

We studied the top-down pressure exerted by nano- and microzooplankton on different prey types at six distinct moments during a spring bloom event, and we assessed how the specific pressure exerted on different prey taxa changed over time. To do so, we conducted dilution experiments (Landry & Hasset, 1982), and analysed the samples through the Utermöhl method for phytoplankton and microzooplankton (Utermöhl, 1958), and through fluorescence *in-situ* hybridization (FISH) for bacterioplankton (Amann & Fuchs, 2008), thereby ensuring a fine taxonomic resolution.

## Sampling site

Our study was conducted at the Helgoland Roads sampling site (54°11.3'N, 7°54.0'E) in the German Bight, North Sea (Wiltshire *et al.*, 2008). This station is located between the islands Helgoland and Düne, approximately 60 km off the coast, and the estuaries of the rivers Elbe and Weser (Franke *et al.*, 2004).

## Sample collection

The abiotic parameters, sea surface temperature, salinity, Secchi depth, nitrate ($NO_3^-$), nitrite ($NO_2^-$), ammonium ($NH_4^+$), phosphate ($PO_4^{3-}$), silicate ($SiO_4^{4-}$) were measured work-daily and dissolved organic carbon (DOC) semi-weekly and were obtained from the Helgoland Roads long-term observation time-series (Wiltshire *et al.*, 2010) of the Biologische Anstalt Helgoland, Alfred-Wegener-Institut, Helmholtz Zentrum für Polar und Meeresforschung.

Biological parameters measured at the site in this study are the phytoplankton and zooplankton species compositions, which were also obtained from the long-term observation series, the chlorophyll content of the water (measured fluorometrically; FluoroProbe, bbe moldaenke) to establish interesting times for the dilution experiments, as well as bacterioplankton counts. Bacterioplankton biomonitoring was performed work-daily from the 17$^{th}$ of March to the 29$^{th}$ of April 2020 by counting 4',6-diamidino2-phenylindole (DAPI) stained 10-mL subsamples using epifluorescence microscopy (see Sidhu *et al.*, 2023 for details).

In order to quantify trophic interactions between zooplankton protists and phytoplankton as well as bacterioplankton we conducted six different dilution experiments on March 24$^{th}$, 31$^{st}$ and April 14$^{th}$, 20$^{th}$, 24$^{th}$ and 28$^{th}$ of 2020. For each experiment, we collected 60 L of seawater at the sea surface (~1 m depth) using a 10 L Niskin bottle. To exclude mesozooplankton, the seawater collected was directly sieved through a 200 $\mu$m nylon mesh and siphoned via a 1 cm wide plastic tube into 20 L containers, letting the water smoothly run by gravity on the container walls to avoid physical damage to organisms (Löder *et al.*, 2010). Notably, here, we focussed on all grazers smaller than 200 $\mu$m in the series of dilution experiments to investigate their direct roles in plankton bloom succession. Namely, Löder *et al.* (2011) illustrated their pivotal role compared to mesozooplankton grazing during a spring phytoplankton bloom event, and we know that in the volumes we are looking at mesozooplankton, such as dominant copepods, play minor roles. Copepods can graze on all groups involved, apart from bacterioplankton (Vargas & González, 2004), i.e., phytoplankton, nanozooplankton and microzooplankton. Also, studying trophic interactions within a complete complex planktonic food web with a high taxonomic resolution complicates drawing conclusions from trophodynamic patterns.

## Seawater processing

From the water sampled, we collected three 250 mL subsamples to determine phytoplankton, nano- and microzooplankton species composition and abundance which were fixed directly with acidified Lugol's iodine solution (10% acetic acid v/v, PanReac AppliChem; with a 1% final concentration v/v; hereafter Lugol's solution) in amber glass bottles, and stored in the dark at 4 ˚C until further analysis. Additionally, we collected bacterioplankton samples for FISH analyses, by pre-filtering 100 mL seawater on 3 $\mu$m polycarbonate filter (47 mm, Isopore$^{TM}$, Merck; bottle top device, NALGENE®), from which we collected a 10 mL subsample that we fixed with formaldehyde (1% v/v, final concentration, Carl Roth) for 1 hour at ambient temperature. Then, we retained this subsample on 0.2 $\mu$m polycarbonate filters (47 mm, Isopore$^{TM}$, Merck) combined with a 0.45 $\mu$m cellulose acetate support membrane filter (Whatman®), which we directly stored at -20 ˚C until further analyses.

To assess seston elemental stoichiometry, we collected samples for two size fractions 0.7-3 $\mu$m (picoseston) and 0.7-200 $\mu$m (total seston). The picoseston was collected as a proxy for the food quality for consumers, and the microseston, which was determined as the difference between the total seston and picoseston, as a proxy for the combined bacterioplankton and phytoplankton quality. More specifically, we filtered, depending on particulate matter loadings in the water column, 200–1,000 mL of the sampled seawater for the two size fractions on pre-combusted and acid-rinsed GF/F filters (25 mm, Whatman$^{TM}$; 5 h at 450 ˚C in the Heraeus muffle furnace; 10% HCl v/v), which we dried and stored in a desiccator (>48 h at 60 ˚C; MEMMERT) until further sample processing.

## Dilution experiment

We conducted a series of six dilution experiments (Landry & Hasset, 1982) to quantify the grazing pressure exerted by nano- and microzooplankton on phytoplankton and

bacterioplankton. Since the suitability of dilution experiments for bacterioplankton has been questioned (Agis *et al.*, 2007; Rychert, 2022), we compared growth rates of total bacterioplankton as well as of individual clades in the 100 % treatments (undiluted) of the incubations and in the field, which indicated that the incubations did not alter the dynamics of bacterioplankton growth (Brüwer *et al.*, 2023). Sequential dilutions of 10, 25, 50 and 100 % of collected seawater were prepared in duplicate in 3 L glass beakers by mixing the seawater sampled with particle-free seawater. Particle-free seawater was obtained from the same collected seawater batch, and filtered on a 0.2 $\mu$m cellulose acetate filter (Sartorius Stedim) mounted on a tripod device (Sartorius Stedim). Then, we gently transferred 1.3-L subsamples without nutrient additions to mimic natural conditions into 1-L polystyrene bottles (Costar®, Corning Incorporated) and placed them on a motorised plankton wheel rotating at a speed of ~3.2 rpm. We incubated these bottles at a light:dark regime of 14:10 h with 20-30 $\mu$mol photons $m^{-2}$ $s^{-1}$ (LED illumination of 446 nm, Mitras Lightbar 2, GHL) in a temperature-controlled room set at the field sea surface temperature (SST) measured during seawater collection. We collected three 250-mL samples of undiluted seawater at the onset of each experiment ($T_0$), and one sample per bottle after 24 h incubation time ($T_{end}$), which we fixed with Lugol's solution and stored as described above.

## Sample analyses

### *Abiotic field parameters*

Salinity, sea surface temperature, and water turbidity (Secchi depth) were measured as described in Wiltshire and Manly (2004). The dissolved nutrient loads (<0.45 $\mu$m; 47 mm, nylon membrane filter, cytiva) of N- pools $NO_3^-$, $NO_2^-$ and $NH_4^+$; of the P-pool $PO_4^{3-}$ and of the Si-pool $SiO_4^{4-}$ were determined with a SEAL Autoanalyzer 3 High Resolution following Grasshoff *et al.* (1999). The acidified DOC (<0.45 $\mu$m fraction; cellulose acetate, Minisart®

Syringe Filter, Sartorius Stedim; 25% HCl v/v, end pH of ~2) was measured in technical duplicate with a TOC analyser (Vario TOC cube Elementar) with potassium hydrogen phthalate as a reference substance. We obtained the copepods counts from the Helgoland Roads data set (Greve *et al.*, 2004; Boersma *et al.*, 2015).We analysed the picoseston and microseston fractions for elemental carbon (C), nitrogen (N) and phosphorus (P) concentrations. C and N were quantified by combustion in a CHNS analyser (Vario MICRO cube, Elementar) combined with the calibration substance Acetanilide ($C_8H_9NO$), and P was determined colorimetrically as orthophosphate via acidic oxidative hydrolysis following Grasshoff *et al.* (1999).

### *Phytoplankton and zooplankton*

We determined phytoplankton, nanozooplankton, and microzooplankton abundance and species composition using the Utermöhl method (Lund *et al.*, 1958; Utermöhl, 1958) by settling, depending on the biomass, 10 to 25 mL of the Lugol's solution samples for 12 h in sedimentation chambers (HYDRO-BIOS). We grouped the phytoplankton species counted into the following six major functional groups: (i) pennate diatoms, (ii) centric diatoms, (iii) silicoflagellates, (iv) coccolithophorids, (v) autotrophic flagellates and (vi) green algae. Similarly, we grouped the different nano- and microzooplankton species in ciliates, heterotrophic nanoflagellates (HNF; 5-20 $\mu$m) and heterotrophic dinoflagellates. Here, we considered all microzooplankton as a heterotrophic group, since mixotrophy is achieved via phagotrophy, keeping ingestion of phototrophic species still possible (Löder *et al.*, 2011).

The biovolume of each plankton species was calculated from cell dimension measurements using geometric formulae following Hillebrand *et al.* (1999) and Harrison *et al.* (2015). Cell volume was converted into carbon following the equations of Menden-Deuer and Lessard (2000) for diatoms (pg C $cell^{-1} = 0.288 \times V^{0.811}$), dinoflagellates (pg C $cell^{-1} = 0.760 \times V^{0.819}$), and other protist plankton (pg C $cell^{-1} = 0.216 \times V^{0.939}$), where V is the cell volume in

$\mu m^3$. Ciliate carbon content was calculated as 0.19 pg C $\mu m^{-3}$, according to Putt and Stoecker (1989).

We divided the phytoplankton group into three size groups based on their individual cellular carbon content: <100 pg C $cell^{-1}$, 100-1,000 pg C $cell^{-1}$ and >1,000 pg C $cell^{-1}$. This division enabled us to determine grazing patterns among phytoplankton cells with distinct volumes belonging to the same taxonomic class. Nano- and microzooplankton division was directly made during species determinations based on the consumer cell seizes with the threshold set at 20 $\mu$m between groups.

### *Bacterioplankton*

To follow the succession of the bacterioplankton during the bloom, the total cell counts (TCC) of bacterioplankton during the bloom were quantified. To assess the successions and abundances within the bacterioplankton, we focussed on three major clades from classes that are known to be dominant at this site, during a spring bloom (Teeling *et al.*, 2012; Teeling *et al.*, 2016): SAR11 clade (SAR11; *Alphaproteobacteria*), *Gammaproteobacteria* (GAM; genus 42a) and *Cytophaga-Flavobacterium* (CF; genus 319a). Bacterioplankton composition and diversity of these clades were identified for the 2$^{nd}$ to 5$^{th}$ field sampling and corresponding dilution experiments (31/03, 14/04, 20/04 and 24/04) by fluorescence *in-situ* hybridization (FISH) with rRNA-targeted probes for all three selected clades [probe GAM42, specific for Gammaproteobacteria (Manz *et al.*, 1992), a mix of probes specific for SAR11 (Gomez-Pereira *et al.*, 2013), and CF319a for Flavobacteria (Manz *et al.*, 1996)]. Bacterioplankton abundances were then converted into biomass using a conversion factor of 20 fg C per cell (Lee & Fuhrman, 1987).

## Data analysis dilution experiment

Phytoplankton and bacterioplankton biomass at $T_0$ and $T_{end}$ were used to compute the growth rates of these groups, as well as the grazing rates of the zooplankton protist community, as described in Landry and Hasset (1982):

$$P_t = P_0 e^{(k-g)\,\Delta t}$$

whereby $P_0$ is the prey biomass at the onset of the dilution experiment and $P_t$ at the end, $k$ is prey growth rate, $g$ is grazing rate, and $\Delta t$ represents the incubation time in day.

We determined the ingestion rate ($d^{-1}$), prey and consumer C concentrations, relative prey stock ($d^{-1}$; %) and prey electivity index (E*) as described in Löder *et al.* (2011). Values of E* cover a range from -1 to 1. E* values of 0 indicate non-selective feeding, between 0 and 1 preference and between -1 and 0 discriminations against prey type. We assessed the linearity of the relationship between dilution levels and prey growth following Calbet and Saiz (2013), and where necessary excluded non-linear responses in the 100% (undiluted) treatments from the analyses, due to feeding saturation and trophic cascades during incubations.

## Statistical analyses

Statistical analyses were performed in R version 4.0.5 (R Core Team, 2021). Linear regression models were fitted for each dilution experiment. Statistical significance was considered when $P < 0.1$. Data visualisations were performed in SigmaPlot version 12.3 (Systat Software Inc., San Jose, CA, USA).

# Results

## Spring bloom succession

*Abiotic field parameters*

During the study period, water temperature increased from 6.3 ˚C on the first sampling day (day 0; 24/03/20) to 8.9 ˚C on the last (day 35; 28/04/20; **Fig. 1A**). The salinity decreased from 33.15 on day 0 to 31.68 on day 35. All macronutrients concentrations - consisting of total dissolved nitrogen, phosphate, and silicate – stayed relatively stable from day 0 to 27, and dropped subsequently in nitrogen and silicate concentrations till day 35 and dropped in phosphate from day 30 till day 35. Notably, the stabilisation of the macronutrients coincided with the drop in salinity from day 2 to 27 (**Fig. 1B,C**). The DOC-pool dropped from day 0 to 16, then rapidly increased till day 30, and dropped again till day 37.

The elemental C/N stoichiometry of the microseston increased from day 0 to 7 during the onset of the main phytoplankton bloom, then declined, subsequently stabilized from day 21 to 31, and then declined further to day 35. The C/P of the microseston remained relatively stable (**Fig. 1D**). The elemental ratios of the picoseston on the other hand was more variable with a lowered C/N and C/P ratios from day 7 to day 31 for C/N followed by an increase till day 35, and to day 21 for C/P (**Fig. 1D**).

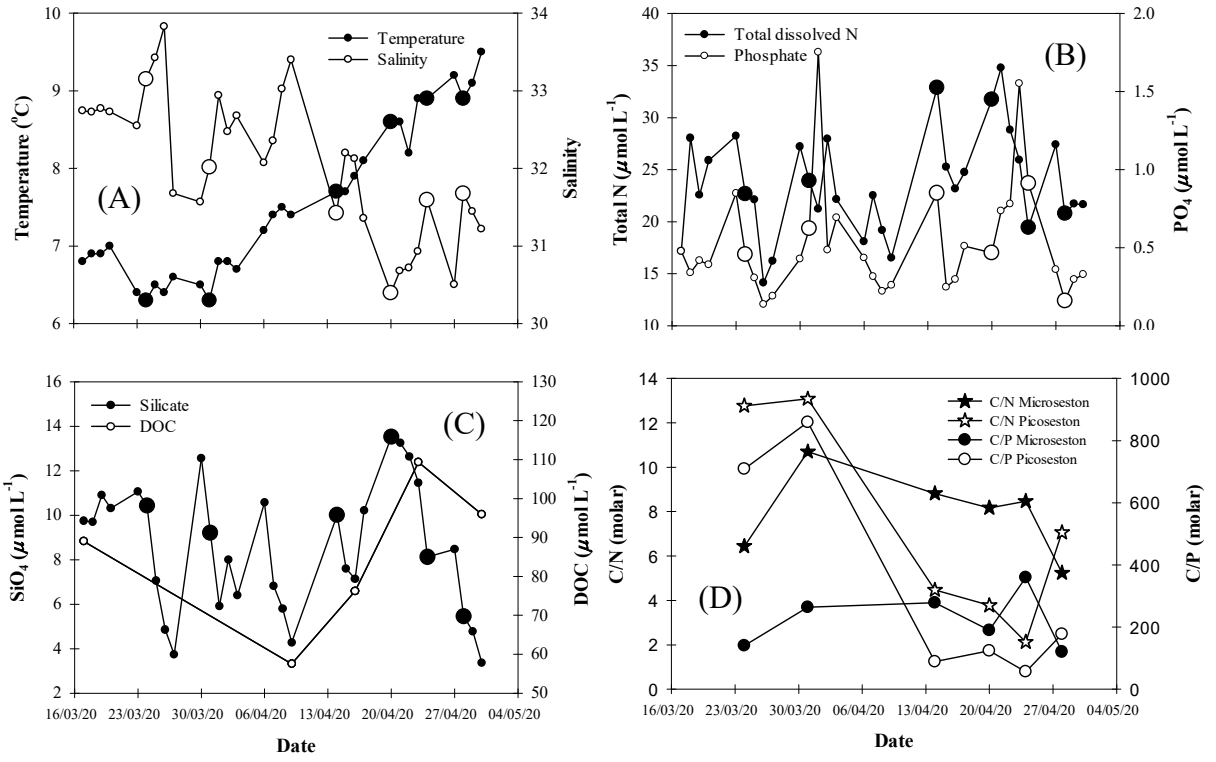

**Figure 1:** Sea surface abiotic parameters of the sampling location Helgoland Roads over the course of the 2020 spring bloom event, (A) dissolved nitrogen and phosphate, (B) dissolved silicate and organic carbon, and (C) sea surface temperature and salinity. Panel (D) presents the elemental stoichiometry of seston for potential food quality assessment of the pico- (3.0 - ~0.7 $\mu$m) and of the microseston (200 – 3.0 $\mu$m). All enlarged symbols represent days at which grazing dilution experiments were conducted.

## *Phytoplankton succession*

The total carbon phytoplankton biomass increased from 4.50 $\mu$g C $L^{-1}$ on day 0 to 46.46 $\mu$g C $L^{-1}$ on day 21, dropped to 11.16 $\mu$g C $L^{-1}$ on day 27, and subsequently increased to 26.40 $\mu$g C $L^{-1}$ on day 35, during a second phytoplankton bloom (**Fig. 2A**). The main phytoplankton bloom consisted of relatively large phytoplankton cells (>1000 pg C $cell^{-1}$), and the second bloom of relatively smaller cells (<100 pg C $cell^{-1}$; **Fig. 2C**). The most dominant phytoplankton class during the main bloom was Mediophyceae with primarily *Dytilum brightwellii* (**SFig. 3A,B**), followed by a post-bloom consisting of species from the classes Bacillariophyceae, Coccolithophyceae and Coscinodiscophyceae. Post-bloom, the chain-forming *Chaetoceros* spp. increased in abundance (**SFig. 3B**).

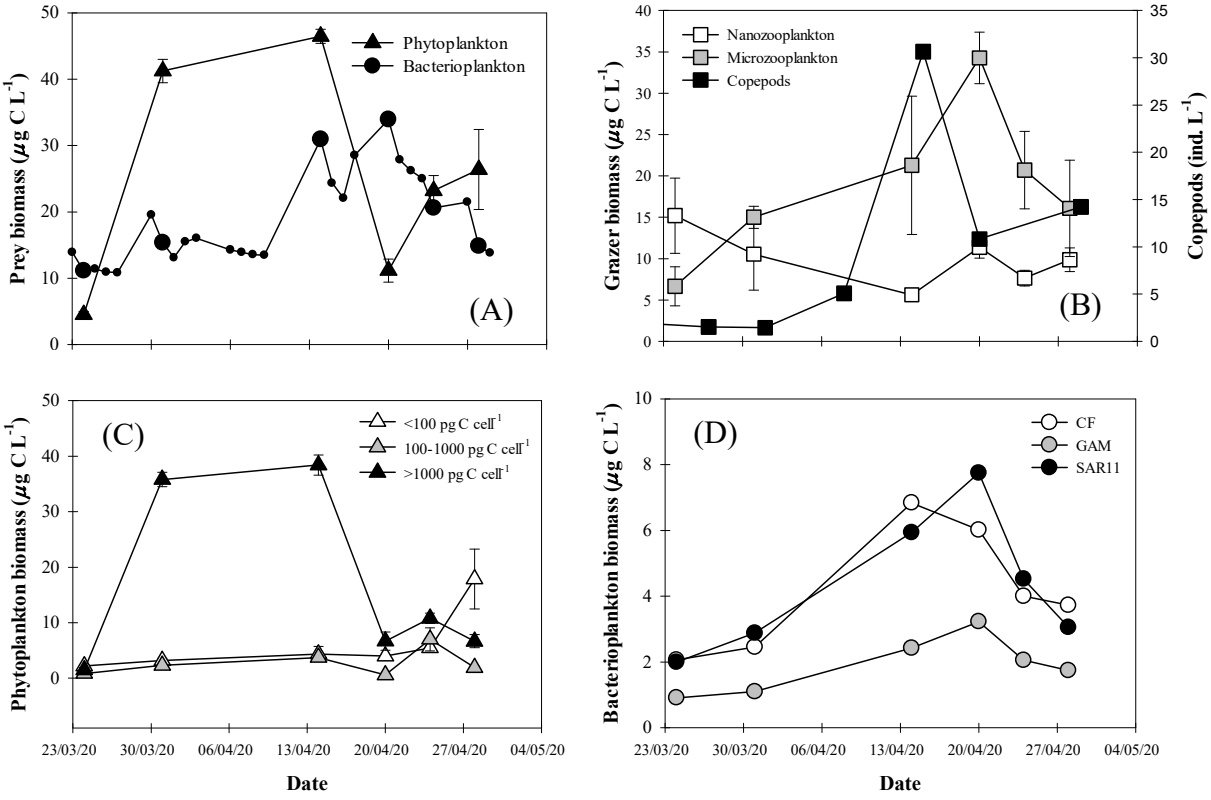


**Figure 2:** Biomass of different plankton taxonomic groups at the sampling location Helgoland Roads over the course of the 2020 spring bloom event, (A) total phytoplankton and bacterioplankton biomass, (B) nanozooplankton and microzooplankton biomass as well as copepod abundances, (C) biomass of phytoplankton size fractions, and (D) biomass of three selected major clades Cytophaga-Flavobacterium (CF), Gammaproteobacteria (GAM) and Alphaproteobacteria SAR11.

## *Bacterioplankton succession*

The bacterioplankton biomass rose from 11.12 $\mu$g C $L^{-1}$ to 33.94 $\mu$g C $L^{-1}$ on day 27, and decreased subsequently to 14.85 $\mu$g C $L^{-1}$ on day 35 (**Fig. 2A**). The bacterioplankton peak coincided with the termination of the main phytoplankton bloom, and with an increase in DOC concentration (**Fig. 1C**). As bacteria are able to utilize and recycle phytoplankton cellular material (Kirchman, 1994), we paid special attention to their succession in relation to the dissolved nutrient loads (**Fig. 1A,B**; **Fig. 2A**; **SFig. 1**). The concentrations of the dissolved N

and Si-pools increased with bacterioplankton biomass (**SFig. 1A,C**), whilst the inorganic P stayed relatively constant and DOC increase delayed when bacterioplankton biomass increased, subsequently P and DOC swiftly increased directly after the termination of the bacterioplankton bloom (**SFig 1B,D**).

Focusing on the three most dominant bacterioplankton clades which represented about 43-58 % of the total counted bacterioplankton cells (TCC), both GAM and SAR11 abundances increased from days 0 to 27 from 0.91 to 3.23 $\mu$g C $L^{-1}$ and from 2.00 $\mu$g C $L^{-1}$ to 7.75 $\mu$g C $L^{-1}$, respectively (**Fig. 2D**). And CF biomass increased from days 0 to 21 from 2.06 $\mu$g C $L^{-1}$ to 6.84 $\mu$g C $L^{-1}$. CF bloomed thus 6 days earlier compared to GAM and SAR11.

## *Nano- and microzooplankton & copepods succession*

Nanozooplankton declined in biomass from 15.19 to 5.62 $\mu$g C $L^{-1}$ from day 0 to 21 (**Fig. 2B**), and subsequently increased in biomass to 9.85 $\mu$g C $L^{-1}$ on day 35. Microzooplankton biomass increased from 6.65 $\mu$g C $L^{-1}$ on day 0 to 34.27 $\mu$g C $L^{-1}$ on day 27, followed by a drop to 16.08 $\mu$g C $L^{-1}$ till day 35. Microzooplankton were dominated by dinoflagellates with an initial biomass of 4.07 $\mu$g C $L^{-1}$ on day 0, which increased to 13.19 $\mu$g C $L^{-1}$ on day 7 concurrently with the rise of phytoplankton. Then, the dinoflagellate biomass stabilized before decreasing to 9.92 $\mu$g C $L^{-1}$ between days 27 and 35, concurrently with the end of the bacterioplankton bloom (**Fig. 2A**; **SFig. 2**). The dinoflagellate assemblage was dominated by species from the taxonomic order *Peridiniales* from day 0 to 35 (**SFig. 4A**). Ciliates had an onset biomass of 2.58 $\mu$g C $L^{-1}$ on day 0 which only increased on day 27 – during the peak of the bacterioplankton bloom – reaching a biomass of 17.89 $\mu$g C $L^{-1}$, and rapidly dropped to 6.16 $\mu$g C $L^{-1}$ on day 35 after the bacterioplankton bloom. The most dominant ciliate order was *Oligotrichida* from day 0 to 35 (**SFig. 4B**).

Copepods were the most dominant mesozooplankton group during the 2020 spring bloom. The adult copepod density started within the study period with 1.53 individuals $L^{-1}$ on

day 3, and increased during the peak of the phytoplankton bloom to 30.66 individuals $L^{-1}$ on day 22 (**Fig. 2B**). Subsequently, copepod abundances decreased rapidly to a density of 10.82 individuals $L^{-1}$ directly after the collapse of the main diatom bloom on day 27, and increased again to 14.22 individuals $L^{-1}$ on day 36, during the second plankton bloom. Potential explanations for the rapid variations in adult copepod densities are for the increase, the simultaneous maturation of younger copepod stages under more favourable environmental conditions (i.e., naupliar and copepodite developmental stages) in the beginning of the bloom, and for the decrease, grazing by higher trophic levels and/or migration.

## Nano- and microzooplankton prey selection and grazing rates

### *Prey selection*

To establish feeding preferences, we computed the electivity index ($E^*$), and we observed that the prey preferences shifted over the bloom's succession (**Table 1**; **Fig. 3**). Zooplankton protists' prey selection within both prey communities shifted over the course of the plankton bloom (**Fig. 3A,B**). At the onset of the phytoplankton bloom on day 0, zooplankton protists preferred intermediate-sized phytoplankton cells (100-1000 pg C $cell^{-1}$) with discrimination against small cells (<100 pg C $cell^{-1}$) and no selection for large (>1000 pg C $cell^{-1}$) cells. This pattern was inverted during the rest of the bloom from day 7 to 35 with a preference for small phytoplankton, and an avoidance of large phytoplankton. Also, on day 31, there was a shift in selection from small phytoplankton to intermediate-sized cells. Regarding the three selected bacterioplankton clades (**Fig. 3B**), there was decreased selection for CF and an increased selection for SAR11 from days 7 to 31. The GAM clade got preferred by the zooplankton protists on day 27, and not preferred on days 7, 21 and 31. Zooplankton protists preferentially consumed total bacterioplankton over total phytoplankton on days 7, 21 and 27, and did not show any preference on day 31 (**Fig. 3C**).

**Table 1:** Calculated parameters and results from linear regression models of the dilution experiments conducted at the sampling location Helgoland Roads over the course of the 2020 spring bloom event. Planktonic growth rate $k$ ($day^{-1}$), nanozooplankton and microzooplankton grazing $g$ ($day^{-1}$), significance $P$-value from linear regression ('****' $P < 0.001$; '***' $P < 0.01$; '**' $P < 0.05$; '*' $P < 0.1$; 'NS' $P > 0.1$), relative loss of phytoplankton or bacterioplankton stock ($day^{-1}$) $P_i$ (%), carbon-specific ingestion rate $I_C$ ($\mu$g C $prey^{-1}$ $day^{-1}$), and electivity index $E^*$ for different phytoplankton taxonomic groups, during the dilution experiments. *MNMC* indicates mean nanozooplankton and microzooplankton carbon biomass.

| **Phytoplankton by Class & Size** | **Experiment 1 (24.03; *MNMC* = 21.84 µg C $L^{-1}$)** | | | | | | **Phytoplankton by Class & Size and Bacterioplankton by Class** | **Experiment 2 (31.03; *MNMC* = 25.53 µg C $L^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k$ | $g$ | $P$ | $P_i$ | $I_C$ | $E^*$ | | $k$ | $g$ | $P$ | $P_i$ | $I_C$ | $E^*$ |
| Bacillariophyceae | 0.71 | 1.39 | ** | 303.22 | 0.05 | 0.11 | Bacillariophyceae | 1.25 | 0.77 | ** | 115.87 | 0.02 | -0.38 |
| - | - | - | - | - | - | - | Bacillariophyta classis incertae sedis | 1.64 | 5.00 | ** | 14692.42 | 0.01 | 0.49 |
| Coccolithophyceae | 0.75 | - | NS | - | - | -0.65 | Coccolithophyceae | 1.04 | 1.59 | *** | 389.10 | 0.10 | -0.04 |
| Coscinodiscophyceae | 0.70 | 1.08 | *** | 195.18 | 0.05 | -0.01 | Coscinodiscophyceae | 1.03 | 1.23 | ** | 240.55 | 0.07 | -0.17 |
| Mediophyceae | 0.87 | 1.73 | ** | 463.84 | 0.12 | 0.22 | Mediophyceae | 0.24 | - | NS | - | - | -0.96 |
| <100 pg C $cell^{-1}$ | 0.60 | - | NS | - | - | -0.40 | <100 pg C $cell^{-1}$ | 1.23 | 1.33 | **** | 277.27 | 0.00 | 0.28 |
| 100-1000 pg C $cell^{-1}$ | 0.92 | 2.12 | *** | 730.78 | 0.05 | 0.19 | 100-1000 pg C $cell^{-1}$ | 0.76 | 0.91 | ** | 147.84 | 0.07 | 0.10 |
| >1000 pg C $cell^{-1}$ | 0.83 | 1.57 | * | 380.91 | 0.08 | 0.05 | >1000 pg C $cell^{-1}$ | 0.24 | - | NS | - | - | -0.99 |
| | | | | | | | CF | 1.16 | 0.86 | **** | 137.12 | 0.05 | -0.06 |
| | | | | | | | GAM | 0.74 | 0.89 | ** | 144.61 | 0.03 | -0.04 |
| | | | | | | | SAR11 | 1.02 | 1.16 | ** | 218.42 | 0.12 | 0.09 |
| **Total Phytoplankton** | **0.85** | **1.42** | ** | **315.08** | **0.25** | **-** | **Total Phytoplankton** | **0.44** | **0.24** | * | **27.46** | **0.38** | **-0.20** |
| | | | | **$I_{C\ total}$** | **4.88** | | | | | | **$I_{C\ total}$** | **11.06** | |
| | | | | | | | **Total Bacterioplankton** | **0.44** | **0.49** | **** | **63.62** | **0.27** | **0.15** |
| | | | | | | | | | | | **$I_{C\ total}$** | **7.75** | |

| **Phytoplankton by Class & Size and Bacterioplankton by Class** | **Experiment 3 (14.04; *MNMC* = 26.90 µg C $L^{-1}$)** | | | | | | **Phytoplankton by Class & Size and Bacterioplankton by Class** | **Experiment 4 (20.04; *MNMC* = 45.64 µg C $L^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k$ | $g$ | $P$ | $P_i$ | $I_C$ | $E^*$ | | $k$ | $g$ | $P$ | $P_i$ | $I_C$ | $E^*$ |
| Bacillariophyceae | 0.78 | - | NS | - | - | -0.57 | Bacillariophyceae | 0.59 | 0.49 | * | 63.90 | 0.02 | -0.16 |
| Bacillariophyta classis incertae sedis | 2.53 | - | NS | - | - | 0.45 | Bacillariophyta classis incertae sedis | 0.20 | - | NS | - | - | -0.38 |
| Coccolithophyceae | 0.49 | - | NS | - | - | 0.14 | Coccolithophyceae | 1.76 | 1.68 | *** | 435.86 | 0.02 | 0.42 |
| Coscinodiscophyceae | 0.69- | - | NS | - | - | -0.25 | Coscinodiscophyceae | 0.64 | - | NS | - | - | 0.07 |
| Mediophyceae | 0.33 | - | NS | - | - | -0.70 | Mediophyceae | 0.16 | - | NS | - | - | -0.63 |
| <100 pg C $cell^{-1}$ | 0.46 | 1.15 | ** | 214.84 | 0.16 | 0.35 | <100 pg C $cell^{-1}$ | 0.80 | 0.77 | **** | 115.65 | 0.06 | 0.44 |
| 100-1000 pg C $cell^{-1}$ | 0.63 | - | NS | - | - | -0.10 | 100-1000 pg C $cell^{-1}$ | 0.92 | - | NS | - | - | 0.00 |
| >1000 pg C $cell^{-1}$ | 0.24 | - | NS | - | - | -0.82 | >1000 pg C $cell^{-1}$ | 0.35 | - | NS | - | - | -0.67 |
| CF | 1.04 | 1.30 | **** | 268.25 | 0.20 | 0.03 | CF | 1.30 | 1.29 | **** | 263.39 | 0.08 | 0.08 |
| GAM | 1.06 | 1.02 | **** | 176.65 | 0.07 | -0.09 | GAM | 1.26 | 1.23 | **** | 241.00 | 0.04 | 0.06 |
| SAR11 | 1.11 | 1.34 | ** | 283.78 | 0.30 | 0.05 | SAR11 | 0.87 | 0.75 | ** | 111.51 | 0.10 | -0.18 |
| **Total Phytoplankton** | **0.40** | **0.45** | * | **56.80** | **0.89** | **-0.12** | **Total Phytoplankton** | **0.30** | **0.45** | *** | **56.25** | **0.094** | **-0.18** |
| | | | | **$I_{C\ total}$** | **20.36** | | | | | | **$I_{C\ total}$** | **4.63** | |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Total Bacterioplankton** | **0.61** | **0.71** | **** | **102.67** | **0.87** | **0.10** | **Total Bacterioplankton** | **0.90** | **0.84** | **** | **130.60** | **0.46** | **0.13** |
| | | | | $I_{C\ total}$ | **19.80** | | | | | | $I_{C\ total}$ | **22.77** | |

| **Phytoplankton by Class & Size and Bacterioplankton by Class** | **Experiment 5 (24.04; *MNMC* = 28.34 µg C L$^{-1}$)** | | | | | | **Phytoplankton by Class & Size** | **Experiment 6 (28.04; *MNMC* = 25.93 µg C L$^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *k* | *g* | *P* | $P_i$ | $I_C$ | *E** | | *k* | *g* | *P* | $P_i$ | $I_C$ | *E** |
| Bacillariophyceae | 1.06 | 1.25 | ** | 247.64 | 0.08 | -0.14 | Bacillariophyceae | 0.21 | - | NS | - | - | -0.52 |
| Bacillariophyta classis incertae sedis | 0.60 | 1.84 | *** | 528.08 | 0.01 | 0.05 | - | - | - | - | - | - | - |
| Coccolithophyceae | 1.68 | 3.65 | *** | 3759.41 | 0.07 | 0.38 | Coccolithophyceae | 1.27 | 2.59 | ** | 1230.45 | 0.13 | 0.11 |
| Coscinodiscophyceae | 0.12 | - | NS | - | - | -0.55 | Coscinodiscophyceae | 1.94 | 4.61 | * | 9973.57 | 0.04 | 0.38 |
| Mediophyceae | 0.58 | - | NS | - | - | -0.21 | Mediophyceae | 0.81 | 0.52 | **** | 67.53 | 0.32 | -0.60 |
| <100 pg C cell$^{-1}$ | 1.42 | 1.88 | *** | 555.28 | 0.26 | 0.02 | <100 pg C cell$^{-1}$ | 0.63 | 1.85 | *** | 536.05 | 0.52 | 0.32 |
| 100-1000 pg C cell$^{-1}$ | 0.70 | - | NS | - | - | 0.25 | 100-1000 pg C cell$^{-1}$ | 0.91 | 0.59 | ** | 79.71 | 0.04 | -0.24 |
| >1000 pg C cell$^{-1}$ | 0.50 | - | NS | - | - | -0.55 | >1000 pg C cell$^{-1}$ | 1.43 | 0.45 | ** | 57.13 | 0.14 | -0.36 |
| CF | 1.27 | 1.50 | **** | 347.99 | 0.07 | 0.16 | | | | | | | |
| GAM | 1.23 | 0.95 | **** | 157.72 | 0.03 | -0.07 | | | | | | | |
| SAR11 | 0.90 | 0.82 | ** | 126.69 | 0.08 | -0.14 | | | | | | | |
| **Total Phytoplankton** | **0.68** | **0.99** | ** | **168.64** | **0.62** | **0.03** | **Total Phytoplankton** | **0.88** | **0.99** | **** | **168.96** | **0.67** | **-** |
| | | | | $I_{C\ total}$ | **19.74** | | | | | | $I_{C\ total}$ | **24.77** | |
| **Total Bacterioplankton** | **0.91** | **0.87** | **** | **137.55** | **0.32** | **-0.03** | | | | | | | |
| | | | | $I_{C\ total}$ | **10.30** | | | | | | | | |

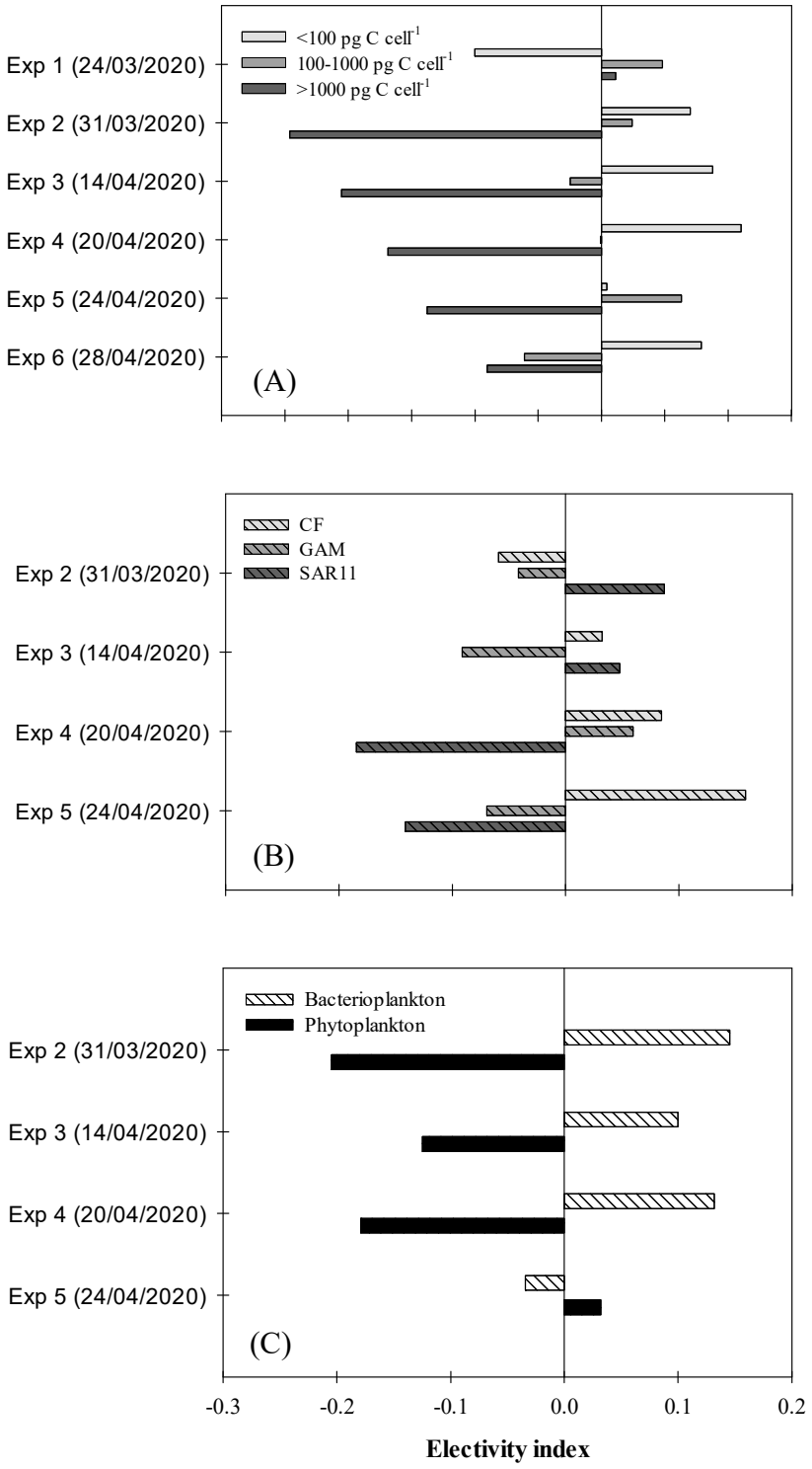


**Figure 3:** Prey preferences of nanozooplankton and microzooplankton at the sampling location Helgoland Roads over the course of the 2020 spring bloom event, (A) within phytoplankton between different size fractions, (B) within bacterioplankton between the selected major clades,

whilst ignoring the rest of the bacterioplankton clades based on FISH, and (C) between the total bacterioplankton based on DAPI stained TCC and total phytoplankton.

## *Grazing rates*

Grazing on all components of the plankton was very high. At the onset of the phytoplankton bloom (*day 0*), zooplankton protists grazed primarily on phytoplankton cells of intermediate size, with a grazing pressure of 730.78 % stock grazed $d^{-1}$ on the size class 100-1000 pg C $cell^{-1}$ (**Table 1**; **Fig. 4A**). *On day 7,* grazing on total phytoplankton was 27.46 % and on total bacterioplankton 63.62 % $d^{-1}$ (**Fig. 4B**). Within the phytoplankton size fractions, the small cells (<100 pg C $cell^{-1}$) were mostly grazed with 277.27 % $d^{-1}$ and no grazing on the large cells (>1000 pg C $cell^{-1}$). From the three bacterioplankton clades, SAR11 was grazed the most with a pressure of 218.42 % $d^{-1}$. *On day 21,* grazing on total phytoplankton was 56.80 % and on total bacterioplankton 102.67 % $d^{-1}$ (**Fig. 4C**). Within the phytoplankton size fractions, there was only grazer pressure exerted on the small cells with 214.84 % $d^{-1}$. Regarding the bacterioplankton clades, the highest grazing pressures were exerted on CF with 268.25 % $d^{-1}$ and on SAR with 283.78 % $d^{-1}$. *On day 27,* grazing on total phytoplankton was 56.25 % and on total bacterioplankton 130.60 % standing stock $d^{-1}$ (**Fig. 4D**). The zooplankton protists exerted only a grazing pressure on the small phytoplankton cells with 115.65 % $d^{-1}$. The grazing pressure on the bacterioplankton clades shifted away from SAR11 towards CF with 263.39 % and GAM with 241.00 % standing stock $d^{-1}$. *On day 31,* grazing on total phytoplankton was 168.64 % and on total bacterioplankton 137.55 % $d^{-1}$ (**Fig. 4E**). The grazing pressure was only on the small phytoplankton cells with 555.29 % standing stock d-1 and was primarily on the CF clade with 347.99 % standing stock $d^{-1}$. *On day 35,* the grazing was primarily on the small phytoplankton cells with 536.05 % standing stock $d^{-1}$ with some pressure on the intermediate-sized cells with 79.71 % and large cells with 57.13 % standing stock $d^{-1}$ (**Fig. 4F**). The grazing

pressure on day 0 was furthermore mainly on phytoplankton cells from the larger-sized taxonomic class Mediophyceae, on day 7 on smaller Coccolithophyceae cells, on days 27 and 31 primarily on Bacillariophyceae and Coccolithophyceae, followed by larger sized phytoplankton cells composed mostly of Mediophyceae on day 35 (**SFig. 5**).

Nano- and microzooplankton grazing pressures on specific bacterioplankton clades shifted over the course of the plankton bloom event. Specifically, the grazing pressure on CF increased with a concurrent abundance drop in CF biomass, whilst we observed a drop in grazing pressure on SAR11 with a concurrent rise in SAR11 biomass between the end of the main phytoplankton bloom (day 21) and the peak of the bacterioplankton bloom (day 27). The grazing pressure on the GAM clade only increased on day 27, which was followed by a sharp decrease in the biomass of this clade.

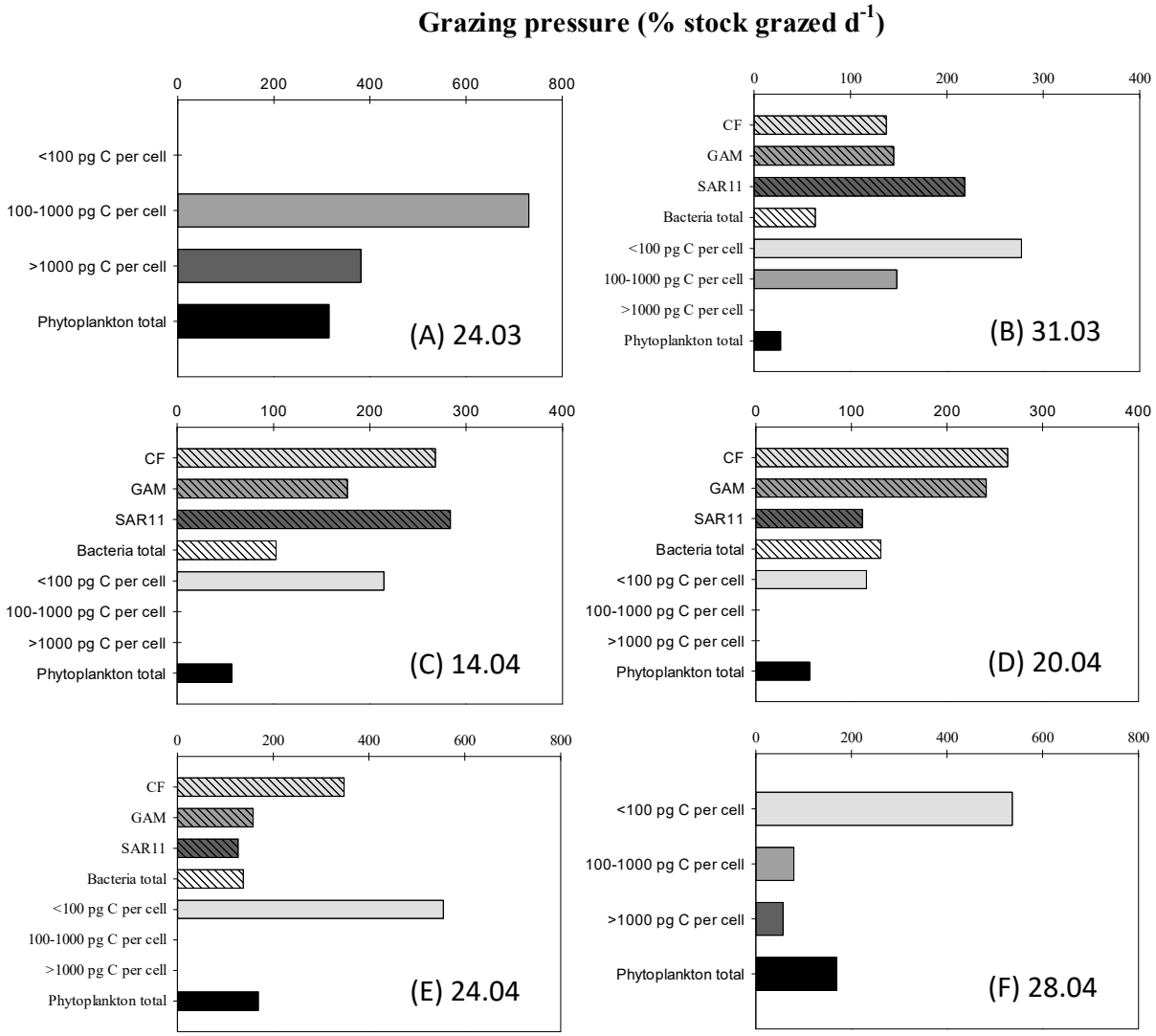


**Figure 4:** Grazing pressure exerted by nanozooplankton and microzooplankton on phytoplankton and bacterioplankton at the sampling location Helgoland Roads determined in grazing dilution experiments at six selected time points over the course of the 2020 spring bloom event.

To evaluate the impact of the grazing by the zooplankton protists on the total phytoplankton and total bacterioplankton standing stocks, we compared the net growth rates of the prey within the dilution bottles and in the field ($K_{field}$) around the sampling days (**Fig. 5**). By comparing both growth rate, we can obtain a proxy for the grazing impacts on phytoplankton and bacterioplankton standing stocks by zooplankton protists during the spring bloom. We

observed for the grazing impact assessment on the phytoplankton stock on all days apart from day 7 a stronger grazing by the zooplankton protists (**Fig. 5A**). The grazing impact by zooplankton protists on bacterioplankton was lower compared to the field (**Fig. 5B**).

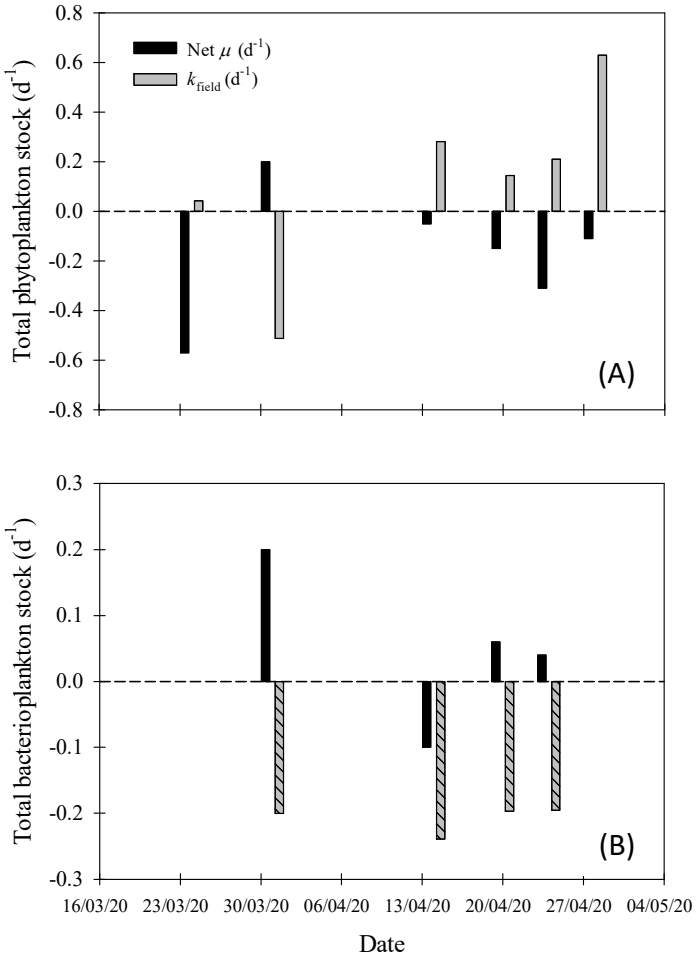


**Figure 5: Evaluation of grazing impacts on the selected prey groups over the succession of the 2020 spring bloom.** In (A) the daily grazing impacts on the total phytoplankton stock and in (B) on the total bacterioplankton stock during the bloom. The net growth in the dilution experiments were determined by subtracting the net apparent grazing rates by the zooplankton protists ($g$) from the gross apparent growth rates ($k$). $K_{field}$ was determined by calculating the growth rate using the adjacent days in the total field count data.

# Discussion

We aimed to quantify the prey selection and grazing pressure of zooplankton protists over the course of a temperate spring bloom event, to evaluate their role in shaping and terminating bloom events of phytoplankton and bacterioplankton. Our data show that nano- and microzooplankton (i) actively shift prey selection within these groups, (ii) shift the grazing pressure between phytoplankton and bacterioplankton based on the dominant grazer's feeding strategies and modes, and (iii) shape phyto- and bacterioplanktonic blooms and substantially contribute to the termination of these blooms.

## Phytoplankton bloom

At the onset of the phytoplankton bloom, nutrient concentrations were high, which enabled rapid microalgal growth. Interestingly, the species that contributed most to this biomass build up was the large chain-forming diatom *Ditylum brightwellii*. Irigoien et al. (2005) and Löder et al. (2011) suggested that microzooplankton grazing shapes the species composition of phytoplankton blooms by exerting a substantial top-down pressure on small phytoplankton which creates a loophole for large, inedible, species to grow. Our results support this hypothesis, as we observed that zooplankton protists preferentially fed on small phytoplankton, and avoided consuming large phytoplankton during most part of the bloom. The high grazing rate on small phytoplankton species at the onset of the bloom (day 7) was likely exerted by Amphidiniales dinoflagellates which were abundant at that time, as this group can efficiently consume small organisms through phagotrophy (Larsen, 1988). It is worth noting that the most abundant microzooplankton taxa, Peridiniales, can feed using a pallium, which enables these dinoflagellates to consume chain-forming diatoms such as *Ditylum brightwellii (Menden-Deuer et al., 2005)*. While this feeding strategy enables Peridinales to thrive in the presence of diatom

chains, these dinoflagellates do not have a high grazing rate (Tillmann, 2004), which may explain that the top-down pressure exerted did not terminate the bloom.

At the onset of a phytoplankton bloom, selective grazing by protists can lead to a bloom of large, inedible species (Irigoien *et al.*, 2005; Löder *et al.*, 2011). Midst a typical spring phytoplankton bloom, dissolved nutrients are depleted, leading to a decrease in phytoplankton biomass (Sommer *et al.*, 2012). However, we observed that the dissolved nutrient concentrations remained relatively high, and seston stoichiometry did not show any sign of nutrient limitation when phytoplankton biomass decreased (i.e., the carbon-to-nutrient ratios did not increase substantially above Redfield; Redfield, 1958). This result implies that nutrients were supplied continuously while phytoplankton biomass increased, which we hypothesize originated from riverine inputs. In support of this hypothesis, the sampling site Helgoland Roads is known to receive dissolved nutrient loads from two major riverine run-offs, the Elbe and Weser (Franke *et al.*, 2004), and we observed a substantial decrease in salinity during the study period suggesting a substantial freshwater input. Interestingly, this implies that the decline of phytoplankton biomass was not caused by nutrient limitation, but rather by top-down pressure exerted by herbivores. This is supported by a concomitant increase in copepod and microzooplankton abundances coinciding with the phytoplankton biomass decline.

After the collapse of *Ditylum brightwellii* marking the end of the first phytoplankton bloom, phytoplankton biomass increased rapidly to form a second bloom dominated by smaller *Chaetoceros* sp. It is noteworthy in this context that *Chaetoceros* sp. are able to take up silicate and other nutrients at low concentrations (Booth *et al.*, 2002), and thus can thrive after blooms of other diatoms. Furthermore, species of *Chaetoceros* sp. nearly all form chains, and the spines characteristic of this genus may increase the effective diameter of a chain of cells, up to five times that of the cells, making them even larger than *D. brightwellii* (Gauld, 1951). Copepods cannot swallow these chains whole, but have to break them up, and the size of diatoms as well as the presence of spines are efficient grazing deterrents (Tollrian & Harvell, 1999). Hence, the

combination of high nutrient affinity and grazing resistance may have enabled *Chaetoceros* sp. to form a second bloom.

## Bacterioplankton bloom

Bacterioplankton blooms typically build up when DOC concentrations increase following a phytoplankton bloom (Teeling *et al.*, 2012), which we observed. When dissolved nutrient concentrations are low, phytoplankton exude substantial amounts of carbohydrates as a mean of getting rid of the excess carbon produced through photosynthesis which cannot be used for growth under nutrient limitation (Obernosterer & Herndl, 1995). However, we did not observe any significant nutrient limitation, and it is likely that additional processes contributed to the increased DOC concentrations. For instance, zooplankton total biomass was highest when DOC concentrations started to increase, and their exudation and respiration may have fuelled the DOC pool. In addition, copepods are sloppy feeders and have been shown to increase substrate availability to bacterioplankton (e.g., Vargas *et al.*, 2007), and the breakdown of *Ditylum brightwellii* may have contributed to the onset of the bacterioplankton bloom. Interestingly, the bacterioplankton bloom responded negatively to dissolved inorganic P and DOC concentrations, which are important substrates needed for bacterial growth (Kirchman, 1994), and was positively correlated with dissolved total N and Si concentrations, suggesting bacterioplankton activity in degrading phytoplankton biomass and remineralising these nutrients. This trophic pathway is an important constituent of the microbial loop which returns DOC to higher trophic levels via its incorporation into bacterial biomass, and is coupled with the classic food chain formed by phytoplankton-zooplankton-nekton (Azam *et al.*, 1983).

The bacterioplankton bloom was accompanied by a rapid increase in the biomass of nanozooplankton and ciliates, which are important bacterioplankton consumers (Yang *et al.*, 2015; Stoecker *et al.*, 2017). Within microzooplankton, we observed a shift in dominance

among dinoflagellate and ciliate orders over the course of the study period, and we suggest that shifts in both feeding modes and prey preference of microzooplankton plays a key role in this highly dynamic interplay of microbial predator-prey interactions. For example, on days 7 and 21, there was a preference for bacterioplankton and high abundance of Gymnodiniales dinoflagellates, which was accompanied by Amphidiniales dinoflagellates on day 7 (**SFig. 4A**). Both taxonomic orders of dinoflagellates are known to eat high abundances of bacterioplankton (Larsen, 1988; Bolch *et al.*, 2011). At the peak of the bacterioplankton bloom (day 27), we observed the highest relative species diversity of ciliates representing the taxonomic orders Cyclotrichiida, Chloreotrichida and Oligotrichida. More specifically, the highest grazing pressure on bacterioplankton coincided with increased ciliate dominance of *Strombidium* spp. and *Laboea* spp. (both genera of the order Oligotrichida) suggesting a trophic link. Furthermore, the decreased top-down pressure on SAR11 coincided with a drop in ciliates of the order Oligotrichida, and the increased top-down pressure on CF coincided with a rise in ciliates of the orders Cyclotrichiida and Choreotrichida during the bloom. These results suggest highly specific interactions, and feeding preferences of zooplankton protists for particular bacterial clades. Consequently, we suggest that the specific top-down pressure exerted on different bacterioplankton clades influences their dynamics. For instance, we observed that the highest grazing pressure on CF on days 21 and 27, coincided with a sharp decrease in CF biomass, while the sharp decrease in grazing pressure on SAR11 between the days 21 and 27, coincided with an increase in SAR11 biomass. To date, the general consensus is that bacterioplankton blooms are shaped by the availability of specific carbon substrate types (Teeling *et al.*, 2012). Our results indicate that while there was utilization of organic matter over the course of the bacterioplankton bloom, there was also strong grazing by zooplankton protists on bacterioplankton. While other mortality causes surely also played a role in terminating the bacterioplankton bloom, the bacterioplankton biomass collapsed when nano- and

microzooplankton biomass and grazing increased, indicating an overall strong top-down pressure.

The evaluation of the daily grazing impacts by zooplankton protists on total phytoplankton and total bacterioplankton standing stocks reveals that the grazing impact by zooplankton protists was higher on total phytoplankton than on total bacterioplankton during the spring bloom. The net *in-situ* growth rates are apart from nano- and microzooplankton grazing (and the other loss factors present <200 $\mu$m) also impacted by mesozooplankton grazing and migration patterns. A potential explanation for the mostly stronger grazing impact by the zooplankton protists on the total phytoplankton standing stock is that mesozooplankton do graze on phytoplankton, bacterioplankton and zooplankton protists to obtain their individual nutritional requirements (Löder *et al.*, 2011; Motwani & Gorokhova, 2013; Boersma *et al.*, 2016). Thus, albeit grazing by zooplankton protist exerts a strong top-down pressure on both phytoplankton and bacterioplankton standing stocks, it needs to be recognized that it does not determine the complete trophodynamics of their prey.

This study illustrates that nano- and microzooplankton exert a significant top-down pressure on phytoplankton and bacterioplankton. Our data show that zooplankton protists can shift their grazing pressure (i) between phytoplankton and bacterioplankton, as well as (ii) within these groups, which (iii) shapes the composition of phyto- and bacterioplankton blooms and substantially contribute to the termination of these blooms. Hence, future studies should integrate the role of zooplankton protists in ecological models and microbial food web studies to get a more profound understanding of these complex interactions which play key roles in microbial food web functioning. This is the first time that we see bacterioplankton bloom termination by grazing.

# Acknowledgements

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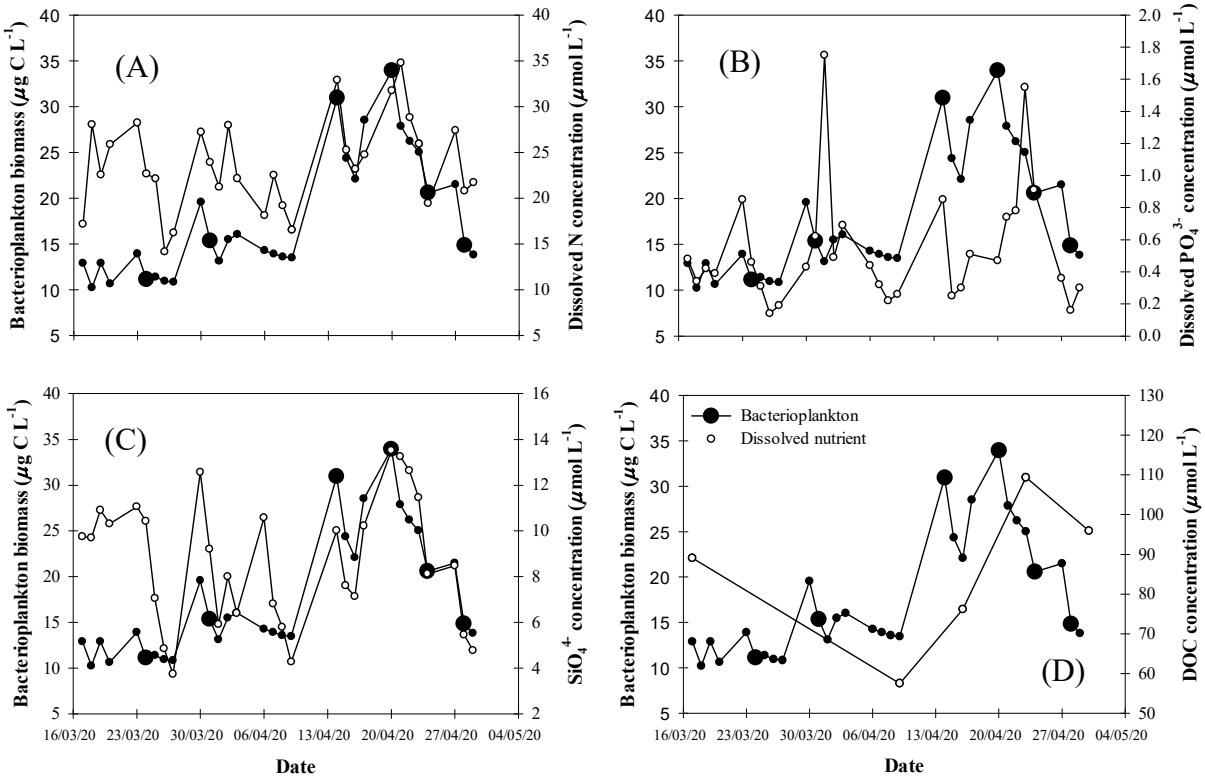
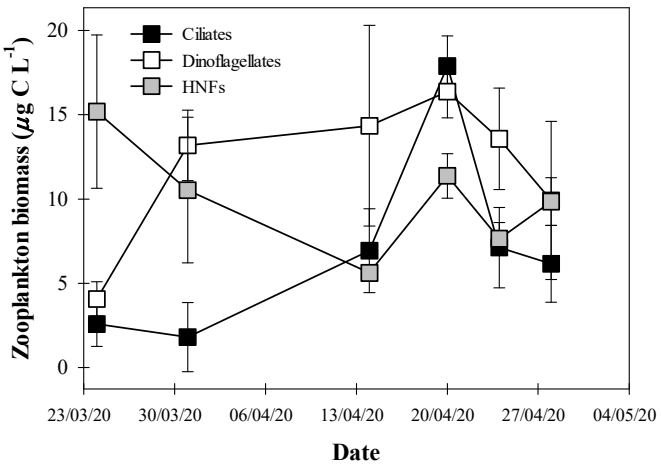
# Supporting material




**SFigure 1:** Bacterioplankton carbon biomass succession in relation to the dissolved nutrients loads of (A) total nitrogen (nitrate, nitrite and ammonium), (B) the inorganic phosphate, (C) silicate and (D) organic carbon (DOC). All enlarged symbols represent the sampling moments used in the dilution experiments.




**SFigure 2:** The succession of the three assessed functional zooplankton groups.

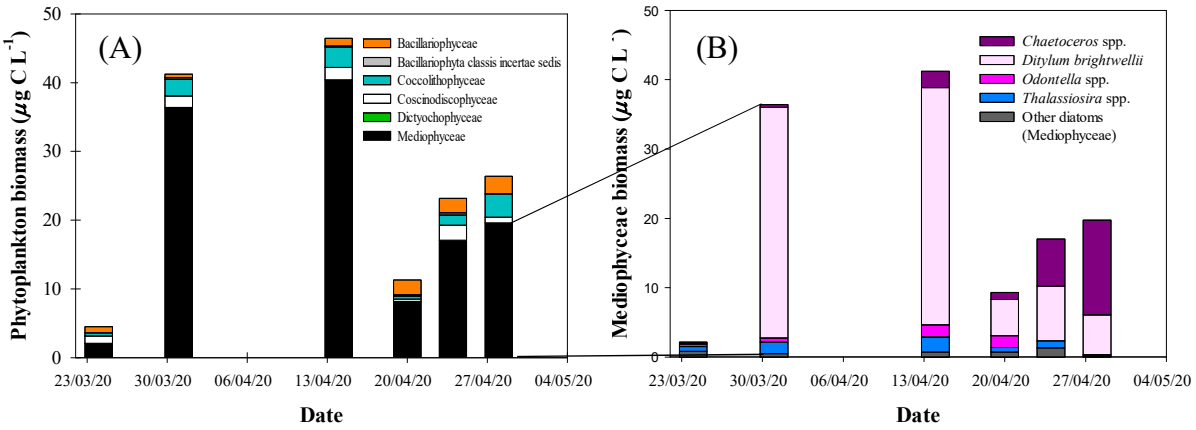
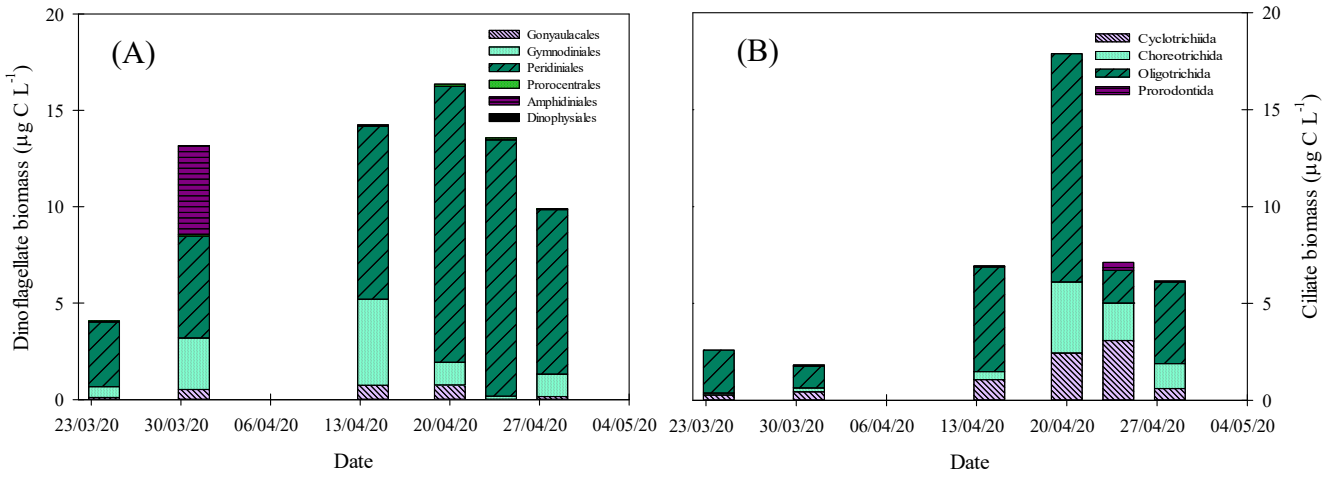
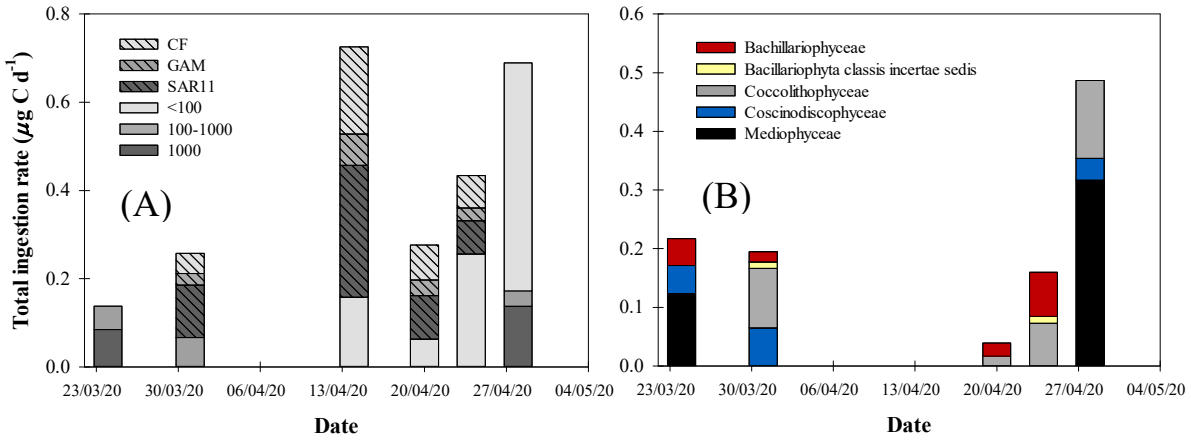



**Figure 3:** Phytoplankton carbon biomass succession per taxonomic (A) classes and (B) genera of the most dominant class Mediophyceae.




**SFigure 4:** Microzooplankton carbon biomass per taxonomic order for (A) dinoflagellates and (B) ciliates.




**SFigure 5:** Total ingestion rates of (A) the assessed prey groups during the series of dilution experiments and (B) per counted major phytoplankton taxonomic class.

# Chapter 3: Nutrient ratios do matter in media

## Thoughts for food: Why are macronutrient ratios of many growth media for phytoplankton so different from the Redfield ratio?

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# Abstract

Phytoplankton cultures used for an array of physiological and ecological studies are created by growing algae and cyanobacteria in water enriched with nutrients. Whilst the relative proportions of nutrients largely influence phytoplankton physiology, with the Redfield ratio (nitrogen/phosphorus of 16/1) often considered as the "optimal" ratio for algal growth, the concentrations and the ratios of added nutrients in published work show enormous variation, even in studies where the aim is to produce high amounts of "healthy" algae. Here, we present the wide variety of molar N/P ratios used in various published growth media, with the aim of increasing awareness of this issue within the scientific community. Especially given the current and predicted changes in relative nutrient availability in many aquatic systems, we recommend a more rigorous consideration of nutrient ratios in growth media, as it may influence the outcome of studies and our capabilities to assess potential futures.

The growth of phytoplankton, and of any other plant, is directly influenced by the environmental availability of nutrients. Liebig's law of the minimum (Von Liebig, 1840) states that the growth and overall production of plants are limited by the least available essential nutrient relative to its demand for growth. Liebig's work has been the foundation for multiple ecological frameworks linking nutrients to growth, including ecological stoichiometry (Sterner & Elser, 2002), which recognises the importance of elemental ratios in driving ecological processes, e.g., phytoplankton growth. Particularly, the ratio of carbon (C) to nitrogen (N) to phosphorus (P); (C/N/P) available in the environment determines growth, and in many cases, phytoplankton growth is highest when nutrient availability is close to the Redfield ratio of 106/16/1 (RR; Redfield, 1958; Loladze & Elser, 2011; Hillebrand *et al.*, 2013; Garcia *et al.*, 2016). Given the importance of nutrients and particularly their ratios for phytoplankton growth, it is surprising that the C/N/P of many growth media used to maintain algal cultures or community assemblages in the laboratory deviate substantially from Redfield values. We obviously realise that in many cases, there are strong scientific reasons for this, e.g., when the study aims to investigate the effects of nutrient limitations or a regional field study without any intended broader implications, but there are also many incidences where such a clear aim is not present.

To assess the diversity in macronutrient compositions of different growth media, we performed an extensive review of the molar N/P ratios in commonly used phytoplankton media. In this study, we discuss the biological and ecological implications of the RR, including its major caveats, and we argue for applying the RR as an average experimental control when composing media in cases where the aim is to have *optimal* growth, and particularly to increase uniformity in experimental controls.

# The Redfield ratio & Why it matters

Studies focussing on open-ocean biogeochemistry have identified that the elemental composition of suspended particulate organic matter (seston) is generally very close to the ratio of dissolved macronutrients, widely known as the RR (Redfield, 1958). So why is this specific ratio important and relevant? Redfield himself suggested that there is a biological basis for this ratio. He explored three potential explanations for what determines the dissolved C/N/P (Redfield, 1958; Williams, 2006; Klausmeier *et al.*, 2008): (i) It was a geochemical coincidence, (ii) phytoplankton have adapted and evolved to resemble the oceans' chemical composition as well as possible, or (iii) the oceans' composition has changed reflecting phytoplankton nutrient requirements via organic processes modulated by phytoplankton activity. The explanation which is supported most in the literature is the latter, that organic processes chemically buffer the ratios of dissolved elements (Falkowski, 2000).

Initially, Redfield proposed that the main mechanism behind this potential explanation is the ongoing competition between nitrogen-fixing and non-fixing phytoplankton (organic processes), which could determine and stabilise the oceanic dissolved N/P ratio, and, therefore seston C/N/P based on the fact that phytoplankton are overall non-homeostatic (Sterner & Elser, 2002). If the oceanic N/P gets too low, N-fixing species (diazotrophic cyanobacteria) would dominate by fixing atmospheric $N_2$, and increase the oceanic dissolved N/P as they diminish and release N-rich matter by decaying, making it bioavailable to oceanic non-fixing species. In cases of N-surplus, denitrification should dominate by both photo- (cyanobacteria) and chemolithotrophs, thus cycling N-surplus back to the atmosphere (Williams, 2006). Five years later, Redfield proposed that those ongoing oscillations in phytoplankton species composition do not only regulate but also set an optimum for both the relative and the absolute compound concentrations (Redfield *et al.*, 1963). He enabled herewith to link biological processes with

the functioning of the whole ocean. Meanwhile, the work has continued, and below we provide two follow-up arguments for this biological basis for the RR.

First, the work by Loladze and Elser (2011) used theoretical considerations to build the optimal living cell with optimal amounts of proteins, carbohydrates and nucleic acids. Here, we define *optimal* growth conditions as thresholds where neither N nor P limits algal growth (Droop, 1974; Rhee, 1978; Thrane *et al.*, 2016). Loladze and Elser linked the canonical N/P=16 to two fundamental biomolecular processes: N-rich protein synthesis and P-rich ribosomal RNA production. Based on these two processes, the N/P is linked to the functioning of the cell, following the biochemical foundations of life (Elser *et al.*, 2000; Falkowski, 2000; Geider & La Roche, 2002; Sterner & Elser, 2002). Both specific processes contribute to the largest N and P pools in most living organisms and cells (Sterner & Elser, 2002), and typically yield a N/P ratio of ~16.

Second, and not surprisingly, considering the building blocks of the optimal cell, experimental work showed that when phytoplankton are growing maximally, their C/N/P ratios are often very close to Redfield (e.g., Goldman *et al.*, 1979; Hillebrand *et al.*, 2013; Garcia *et al.*, 2016; Klip *et al.*, Under review). These works support that there is indeed a direct biological reason for this convergence to the RR. More specifically, these works are based on the chemostat experiment by Rhee (1978), in which he showed via altering the N/P supply ratio whilst keeping the dilution (growth) rate constant that his model chlorophyte *Scenedesmus* sp. lacked homeostasis (in elemental composition) from a supply ratio ranging from 5-80. Goldman *et al.* (1979) continued this chemostat work by altering dilution rates whilst keeping the N/P supply ratio constant using four initial N/P media compositions. They observed that at relatively low growth rates, the algae followed the N/P supply ratio, whilst, at relatively higher growth rates, the elemental compositions converged towards a single N/P, close to Redfield, independent of the type of nutrient limitation. In other words, Goldman *et al.* (1979) noticed that for single unicellular algal populations under continuous culture conditions, there is lower

variation in N/P and C/P ratios, and convergence towards the RR, at higher phytoplankton growth rates. Goldman (1986) suggested furthermore that when another resource (e.g., light, silicate) is limiting than N or P, cellular N/P could stay close to the RR along the entire growth rate gradient. Additionally, Lenton and Klausmeier (2007) found with their model that Redfield's homeostatic mechanism is strikingly robust and would not alter this balance when another resource than P is limiting for N-fixing species. The environment-related phytoplankton growth described above was further studied in a meta-analysis with a whole-taxa approach (i.e., with mono and polycultures from distinct functional groups) by Hillebrand *et al.* (2013), from which the hypotheses arose predicting that the elemental stoichiometric of faster-growing phytoplanktonic populations are approaching the RR combined with lower intercellular trait variabilities, as there are more ways to grow slowly, but only one to grow fast. Recently, we observed that this also holds within one genotype for both convergences to the RR and lowered intercellular trait variabilities at higher growth rates (Klip *et al.*, Under review). Thus, there seems to be a convergence to RR values when phytoplankton grow well, or maybe vice versa, plankton grow well when supplied with N/P=~16/1.

There are several caveats concerning these proposed RR values which need to be addressed. The RR is just an average seston value that is observed in the oceans, which is a function of phylogeny (based on *in-situ* measurements and *optimal* growth conditions), environmental conditions, time and pulse disturbance (Rhee & Gotham, 1980; Klausmeier *et al.*, 2004a; Sterner *et al.*, 2008; Martiny *et al.*, 2013; Garcia *et al.*, 2022). Also, *optimal* cellular N/P (i.e., the transition between N- and P- limitation, critical ratio, $R_c$) is modulated by environmental conditions (i.e., temperature and light climate) and growth rate (Terry *et al.*, 1985; Hillebrand *et al.*, 2013; Thrane *et al.*, 2016; Thrane *et al.*, 2017; Moreno & Martiny, 2018). Despite the influence of spatiotemporal conditions, phylogeny (i.e., type of growth strategy), and type of growth limitation, all phytoplankton cells seem to converge towards a singular value close to the RR at relatively high growth rates (Hillebrand *et al.*, 2013). Also,

according to Elser *et al.* (2022), the RR can be considered an average representation of a balanced nutrient supply ratio for phytoplankton in diverse pelagic ecosystems. So, why do we still use so many different medium recipes?

## Multiple media compositions

There are numerous growth medium recipes available for culturing phytoplankton under controlled conditions in laboratories. These medium recipes vary largely in their N/P, and are rarely 16 (**Fig. 1**). Indeed, studies using different media (without a N/P=~16) to culture phytoplankton and study their (eco)physiology, may assess the responses of suboptimally growing algae. We consider two major cases: (i) The media usage without using "optimal" culturing conditions regarding nutrients to study additive or synergistic effects with other drivers/stressors (e.g., Carneiro *et al.*, 2009; Kremp *et al.*, 2012; Brandenburg *et al.*, 2021); and (ii) experiments aim to assess the effects of distinct media compositions on phytoplankton growth (e.g., Canizares-Villanueva *et al.*, 1995; Dineshkumar *et al.*, 2016; Santhanam *et al.*, 2017), or on food quality for higher trophic levels (e.g., Langer *et al.*, 2019). However, many experiments and approaches aim for optimal algal growth but still do not use Redfield-values-balanced media.

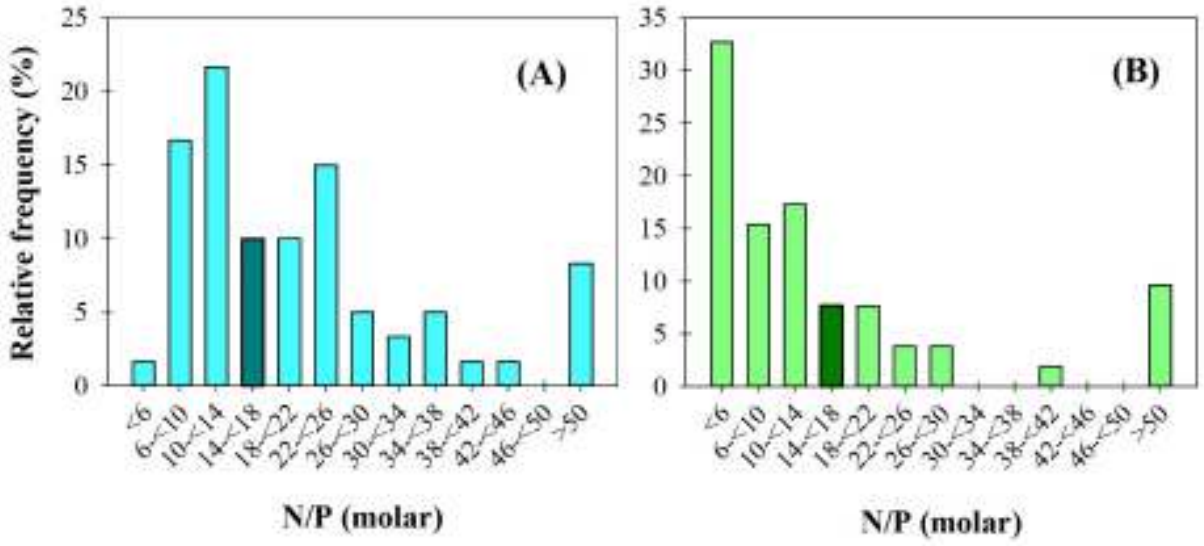


**Figure 1:** Overview of present N/P ratios occurring in frequently used growth media within (A) all assessed seawater-based media presented in **ST1** in blue (n=60), and (B) all assed freshwater-based media in **ST2** in green (n=52). The darker-coloured bars depict the N/P fraction representing the RR.

To better grasp the diversity of macronutrient compositions in media, we reviewed 112 distinct recipes, which we combined into an overview graph (**Fig. 1**) and two supporting literature tables for seawater-based and freshwater-based media (**ST1+ST2**, respectively). Notably, none of the studies presented here aimed to deal with the effects of nutrient ratios on algal growth, and hence all implicitly assumed that they were supplying the algae with nutrients in optimal ratios. We observed a high variety in used dissolved N/P for nutrient enrichment of water to obtain medium. Interestingly, only small fractions of the media investigated here, both for combined seawater-based (10%) and the fresh water-based (8%) media, actually had N/P ratios close to RR (ranging from 14 to <18 molar; **Fig. 1**).

We acknowledge that all media have their own utilities or easiness to use. For example, the medium formulation of Conway (Thompson *et al.*, 1988) and f/2 are applied overall to produce high-density monocultures in laboratories, thus targeting fast growth. Furthermore,

according to Harrison *et al.* (1980), their enriched artificial seawater medium is easy to use. It is simple to prepare and autoclavable without precipitation, can be used for numerous distinct algal species and has a reasonable resemblance to natural seawater. It also could be hard to weigh tiny amounts of individual trace metal salts of not easily obtained chemicals when sometimes preparation time is limited (Trainor *et al.*, 1991). There are quite a few media with, e.g., soil, manure, fertiliser, peat or banana leaf extract additions, which will lead to unclear or unstable N/P afterwards (De Meester & Dumont, 1989; Glazer *et al.*, 1997; Khatun *et al.*, 2006; Tretiak *et al.*, 2021). Lastly, when looking at **ST1** and **ST2**, it becomes quickly clear that most nutrient additions are in round numbers that could be easy to weigh in and compute, despite the fact that this affects the ratios between the nutrients.

Here, we advocate taking macronutrient ratios and RR values into account when preparing a growth medium for phytoplankton, as current experiments are often run under suboptimal growth conditions. This is due to factors such as limitations in the practical work and cutting corners to simplify weighing, which could ultimately lead to results with lessened relevance or lower commercial production yield (e.g., Raoof *et al.*, 2006).

## General recommendation for future experimental media usage

### *Recommended exemplary RR-f/2 medium*

Given the biological importance of elemental ratios, we advocate for more uniformity of the control in the experimental design and to take ratios more into account when choosing experimental conditions. Any deviations in experimental conditions, therefore must discuss not only nutrient supply levels but also supply ratios. We highly recommend using the RR for obtaining algal cultures in a more standardised way. Hence, we suggest making use of a RR-correction in the selected medium recipe for your specific

culturing purpose (e.g., applied by Rokitta & Rost, 2012; Groß *et al.*, 2021), which is also already suggested for dilution grazing experiments (Calbet & Saiz, 2018).

To illustrate how this correction would work, we provide an example of such correction for a commonly used medium for marine and brackish water algal species, RR-corrected f/2 medium (RR-f/2; Guillard & Ryther, 1962; Guillard, 1975), i.e., half strength f medium. We present a RR-corrected medium recipe of f/2 (**Table 1**), which has originally a supply N/P of 24.35 (**ST1**). This f medium is a widely used marine medium, which was cited 8,828 (Guillard & Ryther, 1962) and 6,201 (Guillard, 1975) on Google Scholar (checked on 18.01.2023) and still increasing with also multiple reference omissions, as meanwhile these recipes are considered well-established among peers. We give this RR-f/2 medium as an example for marine sciences, whilst the same medium modification can be performed for other systems, such as for freshwater media for culturing limnic algae.

**Table 1:** Recipe for recommended modifications for macronutrient enrichment to RR-f/2 medium (Redfield, 1958; Guillard & Ryther, 1962; Guillard, 1975) for marine (in particular open sea) phytoplankton cultures.

| **Chemical compound** | **f/2 ($\mu$M)** | **RR-f/2 ($\mu$M)** | **RR-f/2 (mg $L^{-1}$)** |
|---|---|---|---|
| **N-source ($NaNO_3$)** | 883 (882.415) | **882.415** (883) | **75.0** |
| **P-source ($NaH_2PO_4 \cdot H_2O$)** | 36.3 (36.234) | **55.151** (55.188) | **7.610** (7.602) |
| **Si-source ($Na_2SiO_3 \cdot 9H_20$)** | 7.710-15.420 | - | - |
| **$Na_2$·EDTA+** | Ca. 11.7 | 11.713 | 4.36 |
| **$FeCl_3 \cdot 6H_2O$+** | Ca. 11.7 | 11.654 | 3.15 |
| **$CuSO_4 \cdot 5H_2O$** | Ca. 0.04 | 0.0401 | 0.01 |
| **$ZnSO_4 \cdot 7H_2O$** | Ca. 0.08 | 0.106 | 0.022 |
| **$CoCl_2 \cdot 6H_2O$** | Ca. 0.05 | 0.0321 | 0.01 |
| **$MnC_{l2} \cdot 4H_2O$** | Ca. 0.9 | 0.807 | 0.18 |
| **$Na_2MoO_4 \cdot 2H_2O$** | Ca. 0.03 | 0.0248 | 0.006 |

| | | | |
|---|---|---|---|
| **Thiamin·HCl** ($C_{12}H_{17}ClN_4OS{\cdot}HCl$) | 0.3 | 0.297 | 0.1 |
| **Biotin** ($C_{10}H_{16}N_2O_3S$) | 0.002 | 0.00205 | 0.0005 |
| **$B_{12}$** ($C_{63}H_{88}CoN_{14}O_{14}P$) | 0.0004 | 0.000369 | 0.0005 |

We acknowledge that natural *in-situ* measured nutrient concentrations still often deviate from the ones used in media recipes (e.g., Grizzetti *et al.*, 2012; Burson *et al.*, 2016). When investigating, for instance, the impacts of eutrophication or oligotrophication on phytoplanktonic cultures, the nutrient concentrations need to be adjusted accordingly. Additionally, similar ratio regulations in growth media (as the presented RR-correction) could be applicable to other ecosystems within the fields of limnology, pedology and botany (e.g., Cleveland & Liptzin, 2007; Sterner *et al.*, 2008; Fernández-Martínez *et al.*, 2021).

## *Conclusions & Further recommendations*

With only a fraction of microbial species discovered so far, the number of recipes might increase in the future if we continue without considering the RR. It becomes hence increasingly important to assess the specific aim for cultivation and to evaluate the effects of deviating from the RR. Here, we ignored all potential N and P contributions from trace metals, buffers and vitamin pools to the elemental end concentrations because of their relatively low additions to the final medium compositions. There might also be optimisation potential for other parts of the growth medium recipe, as other elemental ratios do also matter and are worth to be studied further and be corrected for in media (e.g., Baines *et al.*, 2010; Mccain & Bertrand, 2021). Also, it is important to realise that by electing certain N- and P-rich buffers to prepare artificial seawater, additional N and P can be added to the final medium composition.

The overall expectation for the future ocean chemistry is, on the one hand, stronger nutrient limitations due to enhanced thermal surface stratification leading to a lowered vertical

supply from deeper (nutrient-rich) water masses (Van De Waal & Litchman, 2020; Martiny *et al.*, 2022), which may actually be linked to deviating N/P ratios, as N-fixing rates potentially increase (e.g., Ward *et al.*, 2013; Hutchins & Fu, 2017). Moreover, because of the declining global stock of non-renewable phosphate rock (globally the main P resource used in agriculture as fertiliser; Cordell *et al.*, 2009) and hence a declining P load entering the ocean via riverine run-off observed in multiple coastal seas (Grizzetti *et al.*, 2012; Peñuelas *et al.*, 2012), we expect that the N/P values will increase. This means that by using, e.g., f/2, we are actually supplying algae with future nutrient ratios rather than those RR ratios that are found at the moment, which hardly represent proper controls of current-day situations. Hence, particularly when assessing phytoplankton attributes (trait values) in global change studies, we suggest using RR-corrected medium recipes as proper experimental controls, as otherwise, multiple drivers/stressors are tested simultaneously (albeit unknowingly). This approach would also facilitate comparisons between regional ecosystems.

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# Supporting material

## Methodology

We assessed distinct medium types often used to illustrate the diversity of N/P molar ratios among commonly used growth mediums. We provide an overview focussing on the purpose and mostly used application(s) of the dissolved nutrient concentrations and the corresponding ratios of the N-source(s) and P-source(s) (**ST1+ST2**). To compile the supplementary literature table, we used book fragments on medium recipes by Andersen *et al.* (2005) and Thompson *et al.* (1988), besides the direct corresponding publications or if needed, modified experimental or industrial usage. We looked for examples of distinct and often used media without using preselected search strings for search engines to illustrate the wide variety of N/P used in media recipes. For this, we selected 112 medium recipes, which we grouped into six categories for: (A) marine algae with 48 media recipes, (B) marine cyanobacteria with 6 media recipes, (C) freshwater algae with 31 media recipes, (D) freshwater cyanobacteria with 13 media recipes, (E) commercial usage and aquaculture research with 6 media recipes, and (F) combinations of categories with 8 media recipes. Noteworthy, all the media compositions presented here are considered good media for their purpose. Lastly, when searching for all the original medium recipes, we came across many modifications and optimisations for specific situations or algae from certain regions without directly considering the macronutrient ratios.

**STable 1:** Overview of distinct seawater-based media used in marine sciences for fundamental and commercially applied research, including their main inorganic macronutrient concentrations in micromolarity ($\mu$M) and ratios in molar. All media are grouped into six types with the following ones for seawater-based recipes: (A) marine algae, (B) marine cyanobacteria, (E) commercial usage and aquaculture and (F) remaining combinations. All macronutrient ratios and concentrations based on recipes showed in Andersen *et al.* (2005) are indicated with ACT and in Thompson *et al.* (1988) with CCAP. When there is no macronutrient addition needed according to the growth medium recipe, it is indicated with NA for not applicable.

| Medium | Group | Composition | Application | N-source | P-source | Si-source | N/P | N/Si | Si/P | Reference(s) |
|---|---|---|---|---|---|---|---|---|---|---|
| **Aquil** | A | Artificial enriched seawater | Marine algae, for trace metal studies | **100** | **10** | 13 | **10.00** | 8.00 | 1.25 | (Morel *et al.*, 1979) |
| **Aquil** | A | Artificial enriched seawater | Marine algae, for trace metal studies | **300** | **10** | 100 | **30.00** | 3.00 | 10.00 | (Price *et al.*, 1989) |
| **Aquil*** | A | Modified artificial enriched seawater | Marine algae, for trace metal studies | **100** | **10** | 100 | **10.00** | 1.00 | 10.00 | ACT: (Morel *et al.*, 1979; Price *et al.*, 1989) |
| **ASN-III** | B | Artificial enriched seawater | Marine cyanobacteria | **8,824** | **88** | NA | **100.70** | NA | NA | (Rippka *et al.*, 1979) |
| **ASP + ASP-2,6,7,12,M** | A | Artificial enriched seawater | Marine algae | **1,000** | **100** | 200 | **10.00** | 5.00 | 2.00 | (Mclachlan, 1964) |
| **ASP-2 +NTA** | A | Artificial enriched seawater | Marine algae | **588** | **29** | 53 | **20.49** | 11.14 | 1.84 | ACT: (Provasoli *et al.*, 1957) |
| **ASP-M** | A | Artificial enriched seawater | Common marine macro and microalgae | **1,000** | **100** | 100 | **10.00** | 10.00 | 1.00 | ACT: (Mclachlan, 1964; Goldman & Mccarthy, 1978) |
| **ASW-III** | A | Artificial enriched seawater | Marine algae | **1,000** | **100** | 200 | **10.00** | 5.00 | 2.00 | (Mclachlan, 1964) |
| **BG (Blue-Green)** | A | Enriched synthetic medium | Marine algae | **176,483** | **17,527** | NA | **10.07** | NA | NA | CCAP |
| **Modified Castenholz D** | B | Enriched artificial seawater medium | Halophilic cyanobacteria | **9,125** | **782** | NA | **11.67** | NA | NA | (Brock, 1976)<br>Source: (Waterbury & Stanier, 1981) |
| **CCAP Artificial Seawater** | A | Enriched artificial seawater medium | Marine algae | **1,320** | **53** | NA | **24.72** | NA | NA | ACT: (Tompkins *et al.*, 1995) |
| **Modified Chu-11** | B | Enriched seawater medium | Marine cyanobacteria | **17,648** | **175** | 204 | **100.70** | 86.50 | 1.20 | (Cohen, 1975)<br>Source: (Waterbury & Stanier, 1981) |
| **bioConway** | A | Enriched artificial seawater medium | Marine microalgae | **1,176,554** | **166,700** | NA | **7.10** | NA | NA | (Tompkins *et al.*, 1995)<br>Source: (Dineshkumar *et al.*, 2016) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **E / Erd-Schreiber** | A | medium E is Erdschreiber medium enriched with soil extract | Marine mono- and dinoflagellates | **47,062** | **1,675** | NA | **28.10** | NA | NA | (Rao, 1980) |
| **Enriched natural seawater (ES)** | A | Enriched natural seawater medium | Marine | **824** | **46** | NA | **17.80** | NA | NA | ACT: (Provasoli, 1968) |
| **ES-T.T. (*Tetraselmis tetrathele*)** | E | Enriched seawater medium, modified from F medium | Large-scale algal production/Aquaculture | **1,765** | **128** | 88 | **13.77** | 20.06 | 0.69 | (Okauchi & Kawamura, 1997) |
| **ESAW** | A | Enriched artificial seawater medium | Coastal and open ocean phytoplankton | **549** | **21.8** | 106 | **25.20** | 5.20 | 4.85 | (Harrison *et al.*, 1980) |
| **ESAW-modified** | A | Enriched artificial seawater medium | Coastal and open ocean phytoplankton | **549** | **22** | 106 | **24.51** | 5.18 | 4.73 | ACT: (Harrison *et al.*, 1980; Berges *et al.*, 2001) |
| **ESNW** | A | Enriched natural seawater medium, modified from ES medium | Marine phytoplankton | **549** | **22** | 106 | **24.50** | 5.18 | 4.73 | ACT: (Harrison *et al.*, 1980; Berges *et al.*, 2001) |
| **F (Guillard's f)** | A | Enriched natural seawater | Marine algae | **1,765** | **73** | NA | **24.34** | NA | NA | (Guillard & Ryther, 1962) |
| **f/2** | A | Enriched natural seawater | Coastal marine algae, especially diatoms | **883** | **36** | 54-107 | **24.33** | 8.25-16.35 | 1.49-2.95 | (Guillard, 1975) |
| **F2P** | E | Pre-assembled powdered medium, based on Guillard's f/2 | Mass culture, marine | **883** | **36** | 54-107 | **24.33** | 8.25-16.35 | 1.49-2.95 | Cell-Hi, Algal Nutrient Media, Varicon aqua |
| **FE** | A | (50% E medium + 50% F medium) | Marine phytoplankton | **24,414** | **874** | NA | **27.90** | NA | NA | (Rao, 1980) |
| **Föyn's "Erdschreiber"** | A | Enriched seawater medium with soil extract | Marine algae, especially diatoms | **1,177** | **141** | NA | **8.35** | NA | NA | (Gross, 1937) |
| **General Purpose (GPM), modified** | A | Soil-water enriched reduced-salinity natural seawater medium, derived from Erd-Schreiber's | Marine algae, especially dinoflagellates | **1,980** | **201** | NA | **9.85** | NA | NA | ACT: (Sweeney *et al.*, 1959; Loeblich, 1975) |
| **h/2** | A | Enriched seawater | Marine algae | **500** | **36** | 54-107 | **13.77** | 4.67-9.26 | 1.49-2.95 | (Guillard, 1963; Guillard, 1975) |
| **I / Drebes** | A | Modified for marine algae (*Stephanopyxis turris*) | Marine diatoms | **500** | **30** | 200 | **16.67** | 2.50 | 6.67 | (Von Stosch & Drebes, 1964) |
| **IWA (SWI)** | A | Iwasaki's enriched natural seawater | Marine algae | **714** | **65** | NA | **11.04** | NA | NA | (Iwasaki, 1961) |
| **IWA (SWII)** | A | Iwasaki's enriched natural seawater | Marine algae | **714** | **67** | NA | **10.60** | NA | NA | (Iwasaki, 1961) |
| **K** | A | Enriched natural seawater media, based on Guillard's f/2 | Brackish, marine phytoplankton | **933** | **10** | 54 | **93.30** | 18.40 | 5.40 | (Keller *et al.*, 1987) |
| **Ketchum & Redfield's** | A | Enriched natural seawater | Marine algae, especially diatoms | **1,098** | **56** | NA | **19.68** | NA | NA | (Ketchum & Redfield, 1938) |
| **Killian** | A | Enriched natural seawater | Marine algae | **1,113** | **139** | NA | **7.99** | NA | NA | (Killian, 1911)<br><br>Source: (Provasoli *et al.*, 1957) |
| **L1** | A | Enriched natural seawater | Coastal marine algae | **883** | **36** | 107 | **24.33** | 8.33 | 2.95 | (Guillard & Hargraves, 1993) |
| **Mann and Meyer's** | A | Enriched artificial seawater medium based upon the ASP-2 (Provasoli *et al.*, 1957) | Marine algae, especially *Phaeodactylum tricornutum* | **11,766** | **574** | NA | **20.50** | NA | NA | (Mann & Myers, 1968) |
| **MAV enrichment** | A | Enriched natural seawater | Marine algae | **989** | **57** | NA | **17.23** | NA | NA | (Droop, 1969) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Mix-TX** | A | 1:1 mixture of modified f/2 medium and ESAW, (with omission of $Na_2$-glycerophosphate) | Marine algae, especially to culture algal viruses | **716** | **18** | 80-106 | **39.45** | 6.74-8.97 | 4.40-5.86 | (Guillard & Ryther, 1962; Harrison *et al.*, 1980; Cottrell & Suttle, 1991; Maat *et al.*, 2016) |
| **Miquel** | A | Enriched seawater medium | Marine microalgae | **1,113** | **139** | NA | **7.79** | NA | NA | (Miquel, 1890)<br><br>Source: (Provasoli *et al.*, 1957) |
| **"Miquel sea water" (Allen & Nelson)** | A | Enriched natural seawater | Marine algae | **3,984** | **139** | NA | **28.62** | NA | NA | (Allen & Nelson, 1910)<br><br>Source: (Provasoli *et al.*, 1957) |
| **MN** | B | Enriched natural seawater | Marine cyanobacteria (same macronutrient addition as for ASN-III medium) | **8,824** | **88** | NA | **100.70** | NA | NA | (Waterbury, 1976)<br><br>Source: (Waterbury & Stanier, 1981) |
| **MNK** | A | Enriched natural seawater | Marine open sea phytoplankton, especially coccolithophores | **235** | **7** | NA | **36.00** | NA | NA | ACT: (Nöel *et al.*, 2004) |
| **PC (*Prochlorococcus*)** | F (A+B) | Enriched natural seawater | Open ocean cyanobacteria and other phytoplankters | **100** | **10** | NA | **10.00** | NA | NA | ACT: PC medium by Keller (Andersen *et al.*, 1997) |
| **Plymouth Erd-schreiber (PE)** | A | Soil-water enriched natural seawater, recipe from the CCAP | For maintaining marine algae cultures | **2,350** | **56** | NA | **42.10** | NA | NA | ACT: (Tompkins *et al.*, 1995) |
| **Pro99 (*Prochlorococcus*)** | F (A+B) | Enriched oligotrophic open ocean water | Open ocean cyanobacteria and other higher ammonia concentrations tolerating phytoplankton species | **800** | **50** | NA | **16.00** | NA | NA | ACT: (Moore *et al.*, 2002) |
| **S50** | A | Enriched synthetic seawater | Marine algae | **989** | **57** | NA | **17.23** | NA | NA | (Droop, 1958) |
| **S77** | A | Enriched marine medium | Marine algae (diatoms) | **989** | **57** | 383 | **17.23** | 2.58 | 6.67 | CCAP: (Turner, 1979) |
| **Schreiber's** | A | Enriched natural seawater | Marine phytoplankton, especially diatoms | **2,353** | **128** | NA | **18.35** | NA | NA | (Schreiber, 1927)<br><br>Source: (Santhanam *et al.*, 2017) |
| **SN (*Synchococcus*)** | B | Enriched dilution natural seawater, N-rich | Marine cyanobacteria | **9,000** | **99** | NA | **90.91** | NA | NA | ACT: (Waterbury, 1986) |
| **Sweeney** | A | Enriched natural seawater | Marine algae | **1,998** | **201** | NA | **9.94** | NA | NA | (Sweeney, 1954)<br><br>Source: (Provasoli *et al.*, 1957) |
| **SWM-I** | A | Seawater | Marine algae | **1,000** | **100** | 200 | **10.00** | 5.00 | 2.00 | (Mclachlan, 1964) |
| **TMRL** | A | Enriched seawater medium; Tongkang Marine Research Laboratory (TMRL), with sodium nitrate as N-source | Marine algae | **1,176,554** | **64,100** | 35,187 | **18.35** | 334.40 | 0.055 | Source: (Santhanam *et al.*, 2017) |
| **TMRL** | A | Enriched seawater medium; Tongkang Marine Research Laboratory (TMRL) | Marine algae | **713** | **83** | 8 | **8.56** | 87.10 | 0.098 | Source: (Rajeswari & Balasubramanian, 2014) |
| **TMRL urea** | A | Enriched seawater medium, modified; Tongkang Marine | Marine algae | **730** | **83** | 8 | **8.76** | 89.10 | 0.98 | Source: (Rajeswari & Balasubramanian, 2014) |

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|---|---|---|---|---|---|---|---|---|---|---|
| | | Research Laboratory (TMRL) | | | | | | | | |
| **Tris-acetate-phosphate (TAP)** | A | Enriched freshwater synthetic medium | Marine microalgae, especially *Chlamydomonas* sp. | **7,010** | **2,712** | NA | **2.59** | NA | NA | (Gorman & Levine, 1965)<br><br>Source: (Kim *et al.*, 2019) |
| **Optimized TAP** | A | Enriched natural seawater medium | Marine microalgae, especially marine *Chlamydomonas* sp. | **10,188** | **269** | NA | **37.82** | NA | NA | (Kim *et al.*, 2019) |
| **UTEX Artificial medium** | A | Enriched artificial seawater medium, modified from Brand's ASP2 medium, suitable for axenic cultures; The Culture Collection of Algae from the University of Texas (UTEX) | Marine algae | **12,300** | **370** | NA | **33.24** | NA | NA | UTEX |
| **Von Stosch** | A | Enriched natural seawater, modified from Grund medium | Marine red (macro)algae | **5,000** | **248** | NA | **20.16** | NA | NA | ACT: (Guiry & Cunningham, 1984) |
| **Walne's** | E | Enriched seawater, recommended by the CCAP | Mass culture of marine phytoplankton for feeding shellfish | **1,180** | **128** | NA | **9.22** | NA | NA | ACT: (Walne, 1970) |
| **West and McBridge's Modified ES (SMW)** | A | Derived from ES medium, enriched natural seawater | Marine algae | **453** | **13** | NA | **34.58** | NA | NA | ACT: (West & Mcbride, 1999) |
| **WP** | E | Pre-assembled powdered medium, based on Walne's | Mass culture, marine | **1,180** | **128** | NA | **9.22** | NA | NA | Cell-Hi, Algal Nutrient Media, Varicon Aqua |
| **Yopp et al.** | B | Enriched artificial seawater medium | Halophilic cyanobacteria | **5,742** | **285** | NA | **20.16** | NA | NA | (Yopp *et al.*, 1978)<br><br>Source: (Waterbury & Stanier, 1981) |

**STable 2:** Overview of distinct freshwater-based media used in aquatic sciences for fundamental and commercially applied research, including their main inorganic macronutrient concentrations in micromolarity ($\mu$M) and ratios in molar. All media are grouped into six types with for freshwater based recipes the following: (C) freshwater algae, (D) freshwater cyanobacteria, (E) commercial usage and aquaculture and (F) remaining combinations. All macronutrient ratios and concentrations based on recipes showed in Andersen *et al.* (2005) are indicated with ACT and in Thompson *et al.* (1988) with CCAP. When there is no macronutrient addition needed according to the growth medium recipe, it is indicated with NA for not applicable.

| Medium | Group | Composition | Application | N-source | P-source | Si-source | N/P | N/Si | Si/P | Reference(s) |
|---|---|---|---|---|---|---|---|---|---|---|
| **Alga-Gro Lake-Water** | E | Enriched natural freshwater media, 2x strength BBM | Freshwater algae | **5,880** | **3,442** | NA | **1.71** | NA | NA | ACT: (Carolina Biological Supply Co.) |
| **Allen's *Cyanidium*** | D | Freshwater synthetic medium, modified | Freshwater cyanobacteria (*Cyanidium caldarium*) | **10,000** | **2,000** | NA | **5.00** | NA | NA | ACT: (Allen, 1959; Watanabe, 2000) |
| **AF-6** | C | Freshwater synthetic medium | Freshwater algae (epibiont *Colacium vesiculosum*) | **1,922** | **102** | NA | **18.81** | NA | NA | (Kato, 1982) |
| **AF6, modified** | C | Freshwater synthetic medium | Freshwater algae | **1,925** | **102** | NA | **18.84** | NA | NA | ACT: (Kato, 1982; Watanabe, 2000) |
| **ASM** | D | Enriched freshwater | Freshwater cyanobacteria | **1,000** | **100** | NA | **10.00** | NA | NA | (Gorham *et al.*, 1964) |
| **ASM-1** | D | Enriched freshwater | Freshwater cyanobacteria | **2,000** | **200** | NA | **10.00** | NA | NA | (Gorham *et al.*, 1964) |
| ***Audouinella*** | C | Soil-water enriched freshwater medium with dry peat moss extract | Freshwater red macroalga *Audouinella* | **412** | **16** | NA | **25.28** | NA | NA | ACT: (Glazer *et al.*, 1997) |
| **Bold's Basal (BBM)** | F (C+D) | Freshwater synthetic medium | Freshwater algae and cyanobacteria | **2,940** | **1,721** | NA | **1.71** | NA | NA | ACT: (Bold, 1949; Bischoff & Bold, 1963) |
| **3N BBM** | D | Freshwater synthetic medium; 3x more nitrate | developed to grow shallow brackish and freshwater and moist soil cyanobacteria | **8,820** | **1,721** | NA | **5.12** | NA | NA | (Thomas & Mantes, 1978) |
| **Blue-Green no.11 (BG-11)** | F (B+D) | Freshwater synthetic medium, modified | Freshwater, soil, thermal and marine cyanobacteria | **17,600** | **175** | NA | **100.57** | NA | NA | ACT: (Allen, 1968; Allen & Stanier, 1968; Rippka *et al.*, 1979) |
| **Modified BG-11** | D | Enriched freshwater | Mainly freshwater cyanobacteria | **17,648** | **230** | NA | **76.85** | NA | NA | (Stanier *et al.*, 1971) |
| **Modified Bristol's** | C | Enriched freshwater synthetic medium | Freshwater algae, especially *Scenedesmus* | **2,941** | **1,717** | NA | **1.71** | NA | NA | (Egan & Trainor, 1989) |
| **C (*Closterium*), modified** | C | Freshwater synthetic medium, modified | Freshwater, desmids green algae | **1,624** | **163** | NA | **9.96** | NA | NA | ACT: (Ichimura, 1971; Watanabe, 2000) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **CA** | C | Freshwater synthetic medium, modified | Developed for culturing freshwater desmids, green algae | **1,699** | **98** | NA | **17.33** | NA | Na | ACT: (Ichimura & Watanabe, 1974; Watanabe, 2000) |
| **Chu #10** | F (C+D) | Synthetic medium to mimic lake water, lower phosphate concentration | Freshwater algae & cyanobacteria | **244** | **29** | 205 | **8.50** | 1.19 | 7.14 | ACT: (Chu, 1942) |
| **Chu #10** | F (C+D) | Synthetic medium to mimic lake water, higher phosphate concentration | Freshwater algae & cyanobacteria | **244** | **57** | 205 | **4.25** | 1.19 | 3.57 | ACT: (Chu, 1942) |
| **Half-Strength Chu #10** | F (C+D) | Synthetic medium to mimic oligotrophic lake water | Freshwater algae & cyanobacteria | **122** | **14** | 102 | **8.47** | 1.20 | 7.08 | ACT: (Nalewajko & O'mahony, 1989) |
| **Modified Chu #10** | D | Synthetic medium | Freshwater cyanobacteria | **244** | **57** | 205 | **4.25** | 1.19 | 3.57 | Source: (Gerloff *et al.*, 1950) |
| **COMBO** | F (C+D) | Derived from WC medium | Freshwater cyanobacteria and algae (for feeding) | **1,000** | **50** | 100 | **20.00** | 10 | 2 | ACT: (Kilham *et al.*, 1998) |
| **D** | D | Freshwater synthetic medium | Thermophilic cyanobacteria from hot springs | **9,130** | **782** | NA | **11.68** | NA | NA | ACT: (Sheridan, 1966) |
| **D11** | C | Freshwater synthetic medium | Freshwater macroalgae | **635** | **86.1** | 211 | **7.38** | 3.01 | 2.45 | ACT: (Graham *et al.*, 1982) |
| **Dauta** | D | Freshwater synthetic medium | Freshwater cyanobacteria | **880,255** | **143,535** | NA | **6.13** | NA | NA | (Lemus, 1991)<br><br>Source: (Canizares-Villanueva *et al.*, 1995) |
| **Diatom (DM)** | C | Soil-water enriched freshwater media, modified | Freshwater diatoms | **85.8** | **101** | 228 | **0.85** | 0.38 | 2.26 | (Beakes *et al.*, 1988) |
| **Diatom, modified** | C | Soil-water enriched freshwater media, modified | Freshwater diatoms | **300** | **400** | ~300 | **0.75** | ~1.00 | ~0.75 | ACT: (Cohn & Pickett-Heaps, 1988; Cohn *et al.*, 2003) |
| **DyIII** | C | Freshwater synthetic medium | Freshwater cryptophytes | **298** | **46** | 53 | **6.42** | 5.63 | 1.14 | ACT: (Lehman, 1976) |
| **DY-V** | C | Freshwater synthetic medium | Freshwater algae | **285** | **10** | 49 | **28.51** | 5.78 | 4.93 | ACT: (Andersen, unpublished) |
| **Fitzgerald** | D | Freshwater synthetic medium, modified Chu #10 medium | Freshwater cyanobacteria | **1,459** | **57** | 204 | **25.61** | 7.15 | 3.58 | (Fitzgerald *et al.*, 1952) |
| **Forsberg's Medium II** | C | Freshwater synthetic medium, modified Chu #10 medium | Freshwater Charalean species, macroalgae | **3,390** | **3** | 82 | **1,052.80** | 41.39 | 25.44 | ACT: (Forsberg, 1965) |
| **Fraquil** | C | Freshwater synthetic medium | To study trace metal interactions with freshwater phytoplankton | **100** | **10** | 13 | **10.00** | 8.00 | 1.25 | ACT: (Morel *et al.*, 1975) |
| **HAMGM** | E | Enriched synthetic freshwater medium | Freshwater algae, especially *Chlorella vulgaris*; Highly Assimilable Minimal Growth Medium (HAMGM) | **15,144** | **1,775** | NA | **8.53** | NA | NA | (Hadj-Romdhane *et al.*, 2012) |
| **Jaworski's (JW, JM)** | C | Enriched synthetic freshwater | Freshwater algae | **1,017** | **190** | NA | **5.35** | NA | NA | CCAP: (Warren *et al.*, 1997) |
| **Kuhl** | C | Freshwater synthetic medium | Freshwater green algae (*Chlorella*) | **10,001** | **5,000** | NA | **2.00** | NA | NA | (Kuhl, 1962; Kuhl & Lorenzen, 1964) |
| **Modified M8** | C | Enriched freshwater | Freshwater algae | **7,418** | **1,906** | NA | **3.89** | NA | NA | Source: (Chellamboli & Perumalsamy, 2014) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **MA** | D | Freshwater synthetic medium | Freshwater cyanobacteria, especially *Microcystis* | **1,789** | **163** | NA | **10.98** | NA | NA | ACT: (Ichimura, 1979) |
| **Medium 7** | C | Enriched freshwater synthetic medium | Freshwater algae, especially *Scenedesmus* | **24** | **0.17** | NA | **136.65** | NA | NA | (Egan & Trainor, 1989) |
| **MES Volvox** | C | Freshwater synthetic medium, modified from Volvox | Freshwater algae | **1,000** | **196** | NA | **5.10** | NA | NA | ACT: (Starr & Zeikus, 1993) |
| **MW** | C | Freshwater enriched medium, derived from W medium | Freshwater dinoflagellate *Peridinium* | **689** | **65** | NA | **10.55** | NA | Na | ACT: (Sako *et al.*, 1984) |
| **N-HS** | C | Freshwater synthetic medium, concentrations based on two German lakes | Freshwater green algae, to study calcification in *Phacotus lenticularis* | **1,680** | **4** | 1,640 | **420.00** | 1.02 | 410.00 | (Hepperle & Krienitz, 1997) |
| **N-HS-Ca** | C | Freshwater synthetic medium, modified N-HS | Freshwater green algae | **750** | **50** | 100 | **15.00** | 7.50 | 2.00 | ACT: (Schlegel *et al.*, 2000) |
| **O2** | D | Freshwater mineral medium | Freshwater cyanobacteria, especially *Oscillatoria agardhii* | **5,883** | **144** | NA | **40.99** | NA | NA | (Van Liere & Mur, 1978) |
| **PP (Proteose Peptone)** | C | Enriched natural freshwater | Freshwater algae | **1,978** | **115** | NA | **17.23** | NA | NA | CCAP |
| **S66** | C | Enriched freshwater medium | Freshwater algae | **989** | **57** | NA | **17.23** | NA | NA | CCAP: (Droop, 1961) |
| ***Spirulina*, modified** | D | Freshwater synthetic medium, modified | Freshwater cyanobacteria | **29,400** | **2,870** | NA | **10.24** | NA | NA | ACT: (Aiba & Ogawa, 1977; Schlösser, 1994) |
| **Sueoka - MM** | C | Minimal Medium | Freshwater algae | **935** | **6,779** | NA | **0.14** | NA | NA | (Sueoka, 1960) |
| **Sueoka - HSMM** | C | High Salt Minimal Medium | Freshwater algae | **9,347** | **13,558** | NA | **0.69** | NA | NA | (Sueoka, 1960) |
| **URO** | C | Based on nutrient concentration of Lake Biwa, Japan | Freshwater microalgae | **63** | **13** | NA | **4.77** | NA | NA | ACT: (Nakahara, 1981; Watanabe, 2000) |
| **Volvox** | C | Freshwater synthetic medium | Freshwater axenic algae, especially for culturing *Volvox* | **500** | **163** | NA | **3.07** | NA | NA | ACT: (Provasoli & Pintner, 1960) |
| **VS** | C | Enriched natural river water | Freshwater red (macro)algae | **500** | **18** | NA | **28.57** | NA | NA | ACT: (Gargiulo *et al.*, 2001) |
| **Waris** | C | Enriched natural medium | Freshwater algae | **1,141** | **151** | NA | **7.53** | NA | NA | (Waris, 1953)<br><br>Source: (Nichols, 1973) |
| **WC** | C | Freshwater synthetic medium | Freshwater algae, and modified marine | **1,000** | **50** | 100 | **20.00** | 10.00 | 2.00 | (Guillard & Lorenzen, 1972) |
| **Wright's (Cryptophyte)** | C | Freshwater synthetic medium | Freshwater cryptomonads | **309** | **29** | 205 | **10.75** | 1.51 | 7.13 | (Wright, 1964) |
| **Zarrouk** | D | Freshwater synthetic medium | Freshwater cyanobacteria | **29,414** | **2,871** | NA | **10.25** | NA | NA | (Zarrouk, 1966)<br><br>Source: (Canizares-Villanueva *et al.*, 1995) |

# Chapter 4: Less algal trait variability when growing faster?

## Less variability when growing faster? Experimental assessment of the relationship of growth rate with functional traits of the marine diatom *Phaeodactylum tricornutum*

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# Abstract

Diversity and its drivers and consequences is at the heart of ecological research. Mostly, studies have focused on different species, but if the causes for increases or decreases in diversity are general, the observed patterns should also be observable within genotypes. As previous research shows that there is higher variability in nitrogen to phosphorus ratios (N/P) between slow-growing unicellular algal populations, compared to fast-growing ones, we expected to observe similar patterns within genetically identical strains growing at different rates. We tested this hypothesis in a laboratory experiment performed with a monoculture of the diatom *Phaeodactylum tricornutum*. Using a growth rate gradient obtained with 10 chemostats, we were able to determine the effect of growth rate on the diatom's elemental stoichiometry as well as on selected traits, such as cell size and shape. Our results showed indeed less intercellular variability (in the selected traits assessed on single-cell level) in the faster-growing populations, which was accompanied by a downward trend in bulk N/P ratios. We pose that this higher variability at lower growth rates potentially results in higher variability of the food sources available for higher trophic levels with potential consequences for transfer efficiency of energy and matter in marine food webs.

# Introduction

Biodiversity, and how it is maintained is one of the hot topics in current ecological research. Traditionally, much of the focus in studies on biodiversity was on different species, but recently genetic within-species diversity has attracted considerable attention (e.g., Reusch & Boyd, 2013; Mcgregor & Sendall, 2021). One of the main reasons that diversity has been so thoroughly studied, is its connection with ecosystem stability and food web efficiency (Striebel *et al.*, 2009; Lewandowska *et al.*, 2015). Interestingly, non-genetic within species diversity has been under much less scrutiny, whereby the processes that affect stability and trophodynamics should theoretically also hold when considering this kind of diversity. One of the biological systems which shows a lot of variation depending on external conditions is the planktonic food web. Especially phytoplankton take up nutrients, such as carbon (C), nitrogen (N) and phosphorus (P) separately, and as a result of their lack of homeostasis show a large diversity in nutrient contents, and as a consequence in their own quality as food for higher trophic levels.

Stoichiometric variation is not random, but related to the growth conditions of the algae. For example, Goldman *et al.* (1979) observed that at very high growth rates, variation in N/P ratios among populations of phytoplankton decreased, and suggested that at these high growth rates, the Redfield ratio (Redfield, 1958), with a N/P of 16 acts as a global attractor. A possible explanation for this phenomenon was given by Loladze and Elser (2011), who asserted that the N/P ratio of 16 represents the very core of cellular make-up with typical contributions of proteins (rich in N) and nucleic acids (rich in P). Only if the relative composition of those is optimal, can high growth rates be attained, or only at maximal growth rates does the composition of the organisms approach the Redfield ratio. In contrast, at lower growth rates this optimality cannot be expected, and as a result the variation in N/P ratios at low growth rates is much higher (e.g., Rhee, 1978; Goldman *et al.*, 1979; Geider & La Roche, 2002): there are

many ways to grow slowly, but only one way to grow fast. In other words, there are many ways in which cells can be limited in their growth, but there is only one optimal state in which they can reach their highest (maximum) growth rate, and as a result the diversity of biochemical make-up of primary producers is strongly dependent on their growth rates.

Not only does the variation in N/P decline with growth rate, also the total value of the ratio approaches to Redfield (Redfield, 1958). As stated above, from a stoichiometric perspective, optimal growing conditions are those where neither N nor P are limiting, essentially representing the threshold between N and P limitation (Droop, 1974; Rhee & Gotham, 1980; Thrane *et al.*, 2016). Moreover, the growth-rate-hypothesis (GRH; Elser *et al.*, 1996; Elser *et al.*, 2000; Sterner & Elser, 2002) predicts a higher demand for P when growing fast as there is a higher need for P-rich ribosomal RNA to support elevated protein synthesis rates, leading to plasticity in the cellular N/P and C/P ratios. So far, we know that the GRH likely only holds under P-limiting growth conditions (Tett *et al.*, 1985; Goldman, 1986; Flynn *et al.*, 2010; Moreno & Martiny, 2018). In N-limited systems on the other hand, N/P goes up with growth rate, albeit more constrained compared to P-limited systems (e.g., Klausmeier *et al.*, 2004b; Hillebrand *et al.*, 2013; Garcia *et al.*, 2016). Nevertheless, in the light of global change with predicted increasingly deviating N/P ratios (Peñuelas *et al.*, 2012; Bopp *et al.*, 2013; Moore *et al.*, 2018), the number and degree of natural systems with N- or P-limitation is expected to rise.

Building on the original study of Goldman, the relationship between N/P ratios and growth rate was further studied by Hillebrand and colleagues (2013), who conducted a meta-analysis on 43 data sets of different phytoplankton species from diverse functional groups in both mono- and polycultures. They corroborated Goldman's predictions and observed an overall convergence in the N/P ratio of bulk material approaching the Redfield ratio with increasing growth rate. They found furthermore that there was less variability in cellular N/P ratios among fast-growing microalgal populations than among slow-growing ones.

If indeed the explanation that slower growth rates will allow for a higher plasticity in cellular N/P ratios holds, this should also be visible *within* populations or even *within* monogenetic strains, i.e., in a fast-growing population, the variation in traits between genetically identical single-cells should be lower than the variation between cells in a slower-growing population. Hence, based on the results of Goldman *et al.* (1979) and Hillebrand *et al.* (2013), we hypothesize that, even within single strains, fast-growing cells should resemble each other more than they would at lower growth rates. This has never been directly tested experimentally. So far, only the recent paper by Groß *et al.* (2021) hints in this direction, but in this study the growth rates were not controlled directly, but varied through changes in environmental conditions.

Answering the question above whether the patterns in intercellular trait variability and elemental composition with growth rate would also hold within a single genotype, is not merely a fascinating academic exercise, it also contributes to a deeper understanding of growth (Isanta-Navarro *et al.*, 2022). In other words, when herbivores graze on relatively slow-growing phytoplankton populations with the larger intercellular variability, there will be a broader window for them to pick from when feeding selectively to obtain their species-specific stoichiometric optima (e.g., Meunier *et al.*, 2012b). More importantly, the results could have important repercussions for our understanding of the availability of diverse food as well as of selective feeding by grazers and of the corresponding population dynamics, such as the outcomes of competition during planktonic bloom events with overall elevated growth rates (Rhee & Gotham, 1980), especially as phytoplanktonic traits have different ecological functions, e.g., cell shape for resource acquisition and predator avoidance (Litchman & Klausmeier, 2008).

Here, we experimentally assessed the influence of cellular growth rate of one single nanoalgal strain on the intrapopulation mean and variability of important planktonic traits, such as size, shape and the elemental composition (in bulk) of the cells in a P-limited system. We

assessed trait variability within and across growth rates along a gradient with 10 different growth rates (0.1-1.0 $d^{-1}$). More specifically, using chemostats with 10 distinct dilution rates creates a gradient in environmental cues in nutrient limitation level caused by dissimilar supply rates, which potentially triggers phenotypic plasticity within the monogenetic populations. For this experiment, we selected the ubiquitous model diatom *Phaeodactylum tricornutum* Bohlin. Since there is only one species in the genus *Phaeodactylum*, only the genus name will be used throughout. Our objective for this study was to investigate whether in accordance with the studies by Goldman *et al.* (1979) and Hillebrand *et al.* (2013) faster-growing algal cells have less cell-to-cell trait variability related to their elemental compositions not only in N/P, but also in other traits, such as cell size and pigment content.

# Material and methods

In this study, we cultured the diatom *Phaeodactylum* in ten different chemostats with different growth rates and used a combination of bulk measurements to corroborate the general patterns of change with modified growth rates and individual measurements using flow cytometry and microscopy to assess the variation within cultures. The results from this study provide novel insights into the phenotypic plasticity within single nanoalgal genotypes, besides the potential linkage between growth rate and other functional traits.

## Experimental design

### *Culturing conditions*

For this study, we grew the ubiquitous phytoplankton species *Phaeodactylum tricornutum* strain CCAP 1052/1A in chemostats. This diatom is a polymorphic species with four morphotypes: fusiform, triradiate, oval and the rare cruciform, that is known to be able to reach

relatively high growth rates (e.g., He *et al.*, 2014; Xue *et al.*, 2015). Besides it is one of the most studied diatoms over the last 60 years.

For the culturing of the diatom, medium was created using artificial seawater (Tropic Marine® Sea Salt CLASSIC; aerated overnight for mixing and to restore the carbonate system; salinity of 32), 0.2 $\mu$m sterile-filtered, and subsequently enriched to f/2 medium nutrient concentrations with a N/P molar ratio of 24.3 (Guillard & Ryther, 1962; Guillard, 1975) with the addition of 5 mg $L^{-1}$ sodium fluorosilicate. The light intensity used was 100 $\mu$mol photons $m^{-2}$ $s^{-1}$ with continuous light (LED illumination of 446 nm, Mitras Lightbar 2, GHL). Stock cultures were pre-acclimated to these conditions for at least 10 generations. During the experiment, algal cells were grown in chemostats made of 1-L laboratory glass bottles placed on magnetic stirrer plates (setting 1, ~250 rpm, IKA) combined with gently sterile-filtered and humidified aeration (0.2 $\mu$m, Midisart® 2000, Sartorius Stedim Biotech, Göttingen, Germany & 0.2 $\mu$m 25 mm Acrodisc® CR Syringe Filter, PTFE Membrane, Pall Corporation) in a 20 ˚C temperature-controlled room.

## Experimental setup

We created a gradient consisting of 10 chemostats with different dilution (flow-through) rates, using peristaltic pumps (REGLO Digital, ISMATEC, Germany, 7x ISM831 + 3x ISM834) ranging from 10 to 100 % total volume displacement per day. More specifically, when the cultures reach steady-state, the dilution rate equals the growth rate ($\mu$). Thus, the cultures were kept under different levels of limitation(s) being still in their exponential growth phase. All pumps were calibrated in the days before the start of the experiment as recommended in the manufacturer's manual.

To initiate the differently growing populations, chemostats were inoculated with a 100-mL aliquot of the *Phaeodactylum* stock culture, and subsequently filled with medium to an end volume of 800 mL. The cultures were left for 72 h in the experimental setting to acclimate to

the chemostats, i.e., aeration and gentle magnetic stirring, before starting the medium feed. First, all pumps were set in the middle of the dilution rate range. After another 48 h (5 d post-inoculation), the pumps were adjusted to the desired dilution rates (0.1-1.0 $d^{-1}$). Every other day, an aliquot (< 5 mL) was taken directly from the chemostats, and cell densities were determined using bench-top flow cytometry (BD Accuri C6, Becton Dickinson). When the cell densities of individual chemostats were constant for three consecutive measuring days, we established that steady-state had been reached. Then, the samples were collected after at least another three generations (after observing steady-state conditions for the first time).

For the assessment of the potential linkage between growth rate and other functional traits, we sampled the algae to assess the following traits: elemental composition, pigment content, cell size, internal complexity of the cell shape (hereafter granularity) and morphotype. Due to the methodological challenges of measuring cellular elemental make-up of single-cells, we settled for a combination of single-cell measurements and bulk ones, because not all traits were amenable to individual measurements. Elemental contents were hence measured using bulk (retained on filters) samples, whereas pigment content, cell size, granularity and morphotype were assessed on individual cells, using flow cytometry and microscopy. More specifically, we used the relative values of the flow cytometric measurements (BD Accuri C6) using three detectors with the blue laser (488 nm), combined with morphotype counting from light micrographs.

## Sample collection

At the harvest of the vessel, at least three generations after reaching steady-state, we collected a subsample of < 80 mL (10 %) of the total volume (800 mL). We divided this aliquot as follows: (i) a 2 mL subsample for measuring with the flow cytometer for relative means and variation within cellular pigment content, cell size and cellular granularity; (ii) a minimum of 20 mL for filtering through a 0.2 $\mu$m (25 mm, Minisart® syringe filter, Sartorius Stedim Biotech

GmbH, Göttingen, Germany) and storing at -20 ˚C until further analysis for dissolved nutrients determination; (iii) a varying subsample (based on the cell density) to assess total C, N and P on three filters; and (iv) the left-over for fixing with Lugol's iodine solution (1 % v/v; Sigma-Aldrich; hereafter Lugol's solution) for light microscopic analysis. For the elemental sampling, we utilized pre-combusted GF/F filters (5 h at 450 ˚C in the Heraeus muffle furnace) to retrieve the material. We dried one filter directly at 60 ˚C for later C and N analysis. The other two filters - for P and back-up - we stored in the -80 ˚C freezer, until further analysis. The fixed samples were stored in the dark at 4 ˚C, until further analysis.

## Sample analyses

### *Particulate and dissolved nutrients*

Cellular carbon and nitrogen quotas were determined by combustion in a CHNS-analyser (Vario MICRO cube, Elementar), using Acetanilide ($C_8H_9NO$) as the calibration standard. Phosphorus quota was measured by colorimetric analysis of orthophosphate ($PO_4^{3-}$) obtained by acidic oxidative hydrolysis, according to Grasshoff *et al.* (1999). Dissolved inorganic N sources of nitrate ($NO_3^-$) and nitrite ($NO_2^-$), dissolved $PO_4^{3-}$ were analysed using a SEAL Autoanalyzer 3 High Resolution, according to the methods of Grasshoff *et al.* (1999). We decided to not measure the macronutrient silicate in this study, because this is only utilized by *Phaeodactylum*, when they are in their oval cell shape (e.g., Borowitzka & Volcani, 1978; Brzezinski *et al.*, 1990).

### *Intercellular trait variation assessment*

The intercellular trait variation within cultures of pigment content, cell size and granularity were assessed using the Accuri software CFlow Plus version 1.0.264.15. The flow cytometer is equipped with two lasers, a common air-cooled blue argon-ion laser (488 nm excitation) and a

red diode laser (640 nm excitation), from which we used the first one. After excitation at this wavelength, the autofluorescence of the cellular pigment content emits at a wavelength of > 670 nm (red fluorescence; emission detector FL3; e.g., Marie *et al.*, 2005). This excitation wavelength can detect the pigments chlorophyll *a* and *b*, carotenoids, xanthophyll and peridinin (Rogers *et al.*, 2012), from which chlorophyll *a* and β-carotene are known to be synthesized by *Phaeodactylum* (e.g., Carreto & Catoggio, 1976). To simplify this, we used this autofluorescence signal (obtained by FL3) as a composite estimate of pigment content, most of which is chlorophyll *a*. The scatter detectors were used with forward scatter (FSC; 0˚ ±13) as a proxy for cell size, and side scatter (SSC; 90˚ ±13) as a proxy for cellular granularity. We assessed the cellular granularity, which is a measure for the internal complexity of the cells, i.e., basically of the concentration of material in their cytoplasm.

In the cytograms produced by flow cytometry, the clusters corresponding to the fresh *Phaeodactylum* populations were automatically enumerated by drawing regions around them, which were clearly distinguishable event clusters from the background noise. The number of events within them was noted. We could however not distinguish different morphotypes within such regions. Subsequently, the means and the corresponding relative coefficients of variation (CVs in %; CV=SD/mean*100) of the clusters were noted with plotting FSC against FL3, and for the granularity SSC versus FL3. They were noted both in arbitrary units (a.u.) and in percentages. We used the means and variability reported from the flow cytometer. Our cell size and cellular granularity determinations were based on the mean FSC and SSC counts per algal population of at least 1,000 assessed cells respectively.

The diatom *Phaeodactylum* is a known polymorph, hence an ideal candidate to study intercellular variability and phenotypic plasticity. We assessed the cell morphometry in the subsamples that were fixed with Lugol's solution using a camera (AxioCam HRc, Zeiss) coupled to an inverted microscope (Observer A1, Zeiss) combined with software (Axiovision SE64, Rel. 4.9, Zeiss). The subsamples were transferred into Utermöhl chambers and left for at

least 8 h to settle (Utermöhl, 1958). We determined *Phaeodactylum* cell shapes on at least five light micrographs showing a minimum of 1,000 cells in total for the following cell shapes: fusiform, triradiate, oval and cruciform (e.g., He *et al.*, 2014).

## Statistical analyses

We collected and processed the data for assessment of the intercellular variability of the cells and their potential growth limitations as follows: (i) all the presented CV values were directly obtained from the CFlow Plus software (BD Accuri™, version 1.0.264.15); and (ii) the variability in the morphometric distribution we described by the Shannon-Wiener diversity index (H'; Weaver & Shannon, 1963).

Data visualisations and statistical analyses were performed in the software SigmaPlot version 12.3 (Systat Software Inc., San Jose, CA, USA). All analyses were carried out using an exponential regression type analysis, using the complete gradient of growth rates. A 3-parameter exponential regression was fitted through all the data to allow for a non-linear response to growth rate, as well as the fact that none of the variables measured can become negative, which cannot be avoided with simple linear regressions. Statistical significance was considered when $p < 0.05$.

# Results

## Growth conditions

During harvest, subsamples were collected for dissolved nutrient analyses of $NO_3^-$, $NO_2^-$ and $PO_4^{3-}$. The measured dissolved nutrient concentrations can be found in **S1 Table**, and are in similar ranges as measured in e.g., Ahlgren (1985) and Grosse *et al.* (2017). The cell densities at this moment varied from 600,000 to 1,400,000 cells $mL^{-1}$ with no clear trend in density varitation indicating that the maintained cellular growth rates

were a result of different levels of a limitation - likely P-limitation - as an environmental cue (**S1 Fig.**).

# Functional traits

## *Particulate nutrient ratios*

To determine the cellular elemental stoichiometry within the different chemostats, we measured particulate macronutrients C, N, and P. We observed a downward trend along the growth rate gradient for all three ratios: N/P ($R^2 = 0.70$, $p = 0.0086$), C/N ($R^2 = 0.96$, $p < 0.0001$) and C/P ($R^2 = 0.94$, $p < 0.0001$; **Fig. 1**). These trends suggest that there is an optimal ratio at any growth rate, but that the optimum declines towards Redfield values at high growth rates. With higher growth rates, elemental cellular compositions approached the Redfield ratio.

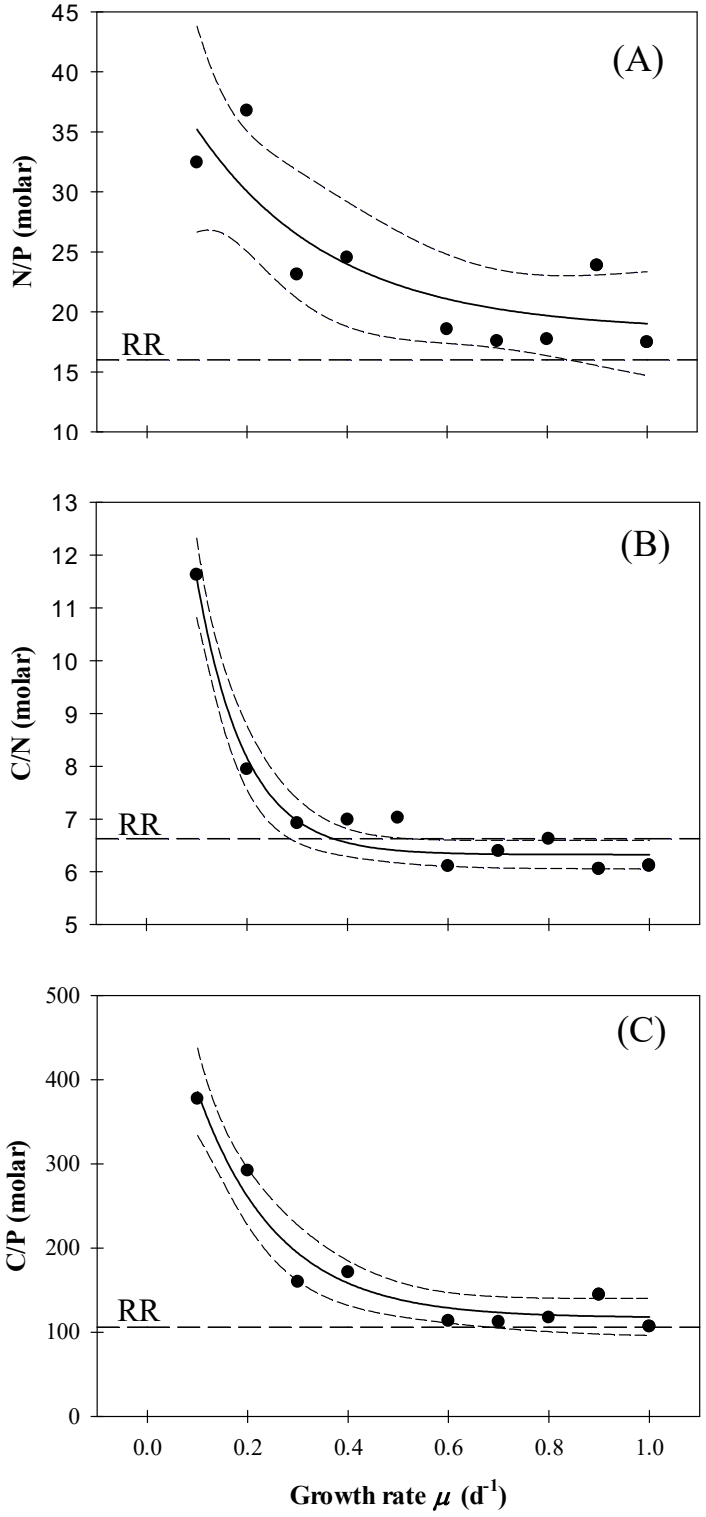


**Figure 1: Cellular particulate macronutrient ratios depicted with black solid circles (A-C) of the N/P (A), C/N (B) and C/P (C) ratios along the growth rate gradient.** We fitted exponential decay regression models for all three ratios. The P content of the 0.5 $d^{-1}$ population was too low with 0.15 pg $cell^{-1}$, which led to unrealistically high N/P and C/P ratios. Hence, the

corresponding values got discarded from all further analyses. We added short-dashed lines depicting the 95% confidence intervals. The black horizontal long-dashed lines represent the Redfield ratio (RR).

## *Intercellular trait variation assessment*

The assessment of the mean and the relative variability of pigment content, cell size, and cellular granularity within one single strain indicated in general that fast-growing populations have indeed lower cell-to-cell variation (**Fig. 2+3**). To aid explaining the intercellular variation assessment via flow cytometry, we included three examples of zoomed-in cytograms and their corresponding event spreads (**Fig. 2**). For the autofluorescent signal representing an estimation of the relative cellular pigment content, there is no significant response in the mean pigment content along the gradient ($R^2$ = 0.36, $p$ = 0.17, **Fig. 3A**), and a significant downward trend in the coefficient of variation from 143.4% for the slowest-growing population to 40.6% for the fastest one ($R^2$ = 0.86, $p$ = 0.0001, **Fig. 3B**). For the relative cell size, there is no significant response in the mean size along the gradient ($R^2$ = 0.018, $p$ = 0.92, **Fig. 3C**), and again a significant downward trend in the cell-to-cell variation from 124.2% for the slowest-growing population to 75.6% for the fastest one ($R^2$ = 0.92, $p$ < 0.0001, **Fig. 3D**). For cellular granularity, we found a statistically significant downward trend in the mean cellular granularity ranging from 378,555 to 54,684 a.u. ($R^2$ = 0.98, $p$ < 0.0001, **Fig. 3E**), and also negative trend in cell-to-cell variation along the growth rate gradient, albeit in this case a non-significant one ($R^2$ = 0.28, $p$ = 0.23, **Fig. 3F**).

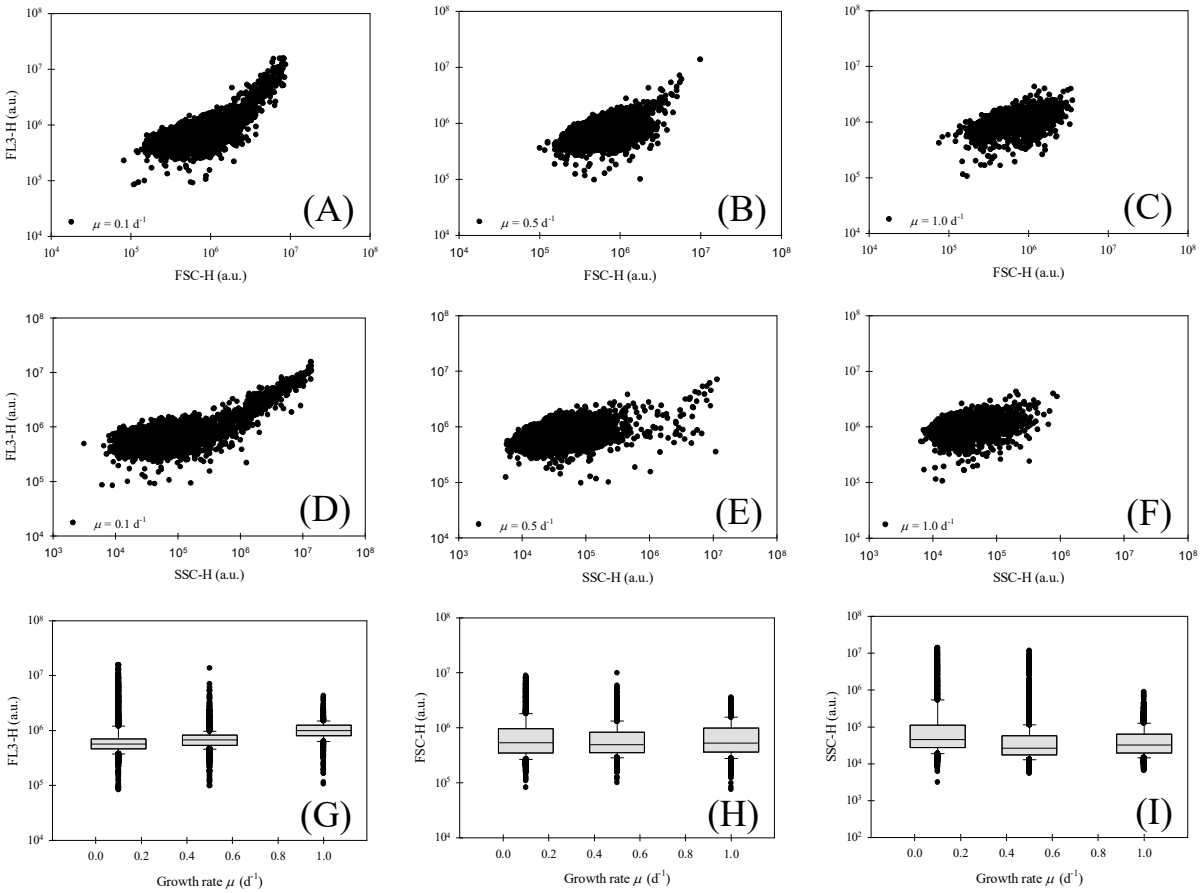


**Figure 2: Three visualisation examples of slow-, intermediate and fast- growing populations using zoomed-in cytograms on *Phaeodactylum* of log-transformed forward scatter (FSC-H; proxy for cell size) over FL3 channel (FL3-H; proxy for grouped pigment content; A-C), of side scatter (SSC-H; proxy for cellular granularity) over FL3 channel (D-F) and the corresponding event spreads including boxplot presentation (G-I).** Each dot represents a registration event by the flow cytometer of a single *Phaeodactylum* cell.

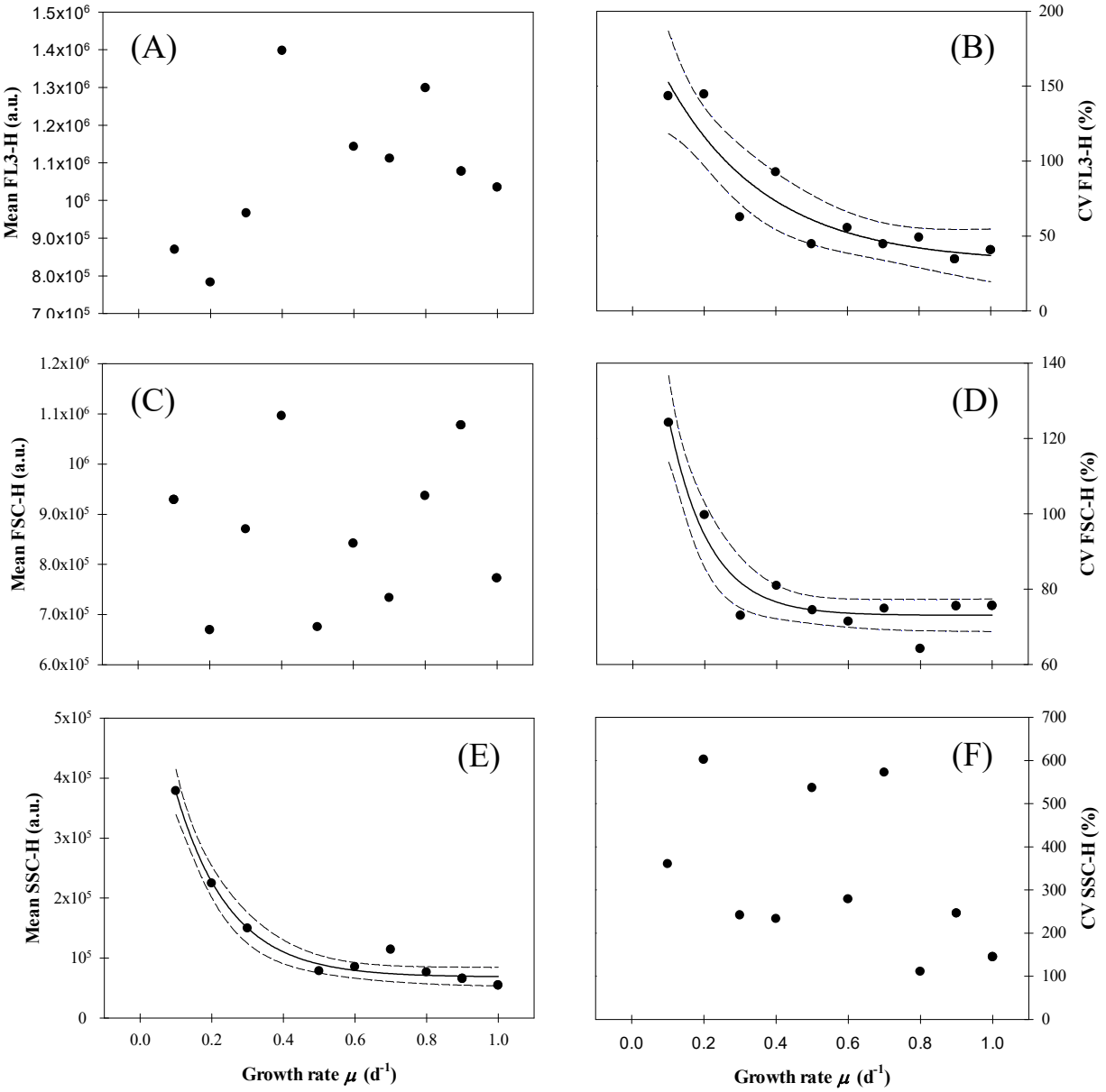


**Figure 3: Assessment of the mean (in arbitrary units; a.u.) and the relative CV (in %) of the pigment content (A+B), cell size (C+D) and cellular granularity (E+F) along the growth rate gradient using exponential regression models.** Each circle represents one chemostat population. Where for mean pigment content an exponential rise to maximum model and for all other plots exponential decay regressions were significant, we indicated this by adding the corresponding regression lines through these measurements combined with 95% confidence intervals showed with the black short-dashed lines. Noteworthy, despite the fact that

all measurements displayed in this figure were detected concurrently by the flow cytometer, we discarded for the mean pigment content and the mean cellular granularity (A+E) all substantially deviating measured values of 720,748 a.u. at 0.5 $d^{-1}$ and 550,059 a.u. at 0.4 $d^{-1}$, respectively, from all further analyses.

## *Cell morphometry*

Light microscopic assessment of the fixed samples showed a minor shift in relative distribution in morphotypes and an increase in cell morphological diversity in faster-growing populations (**Fig. 4**). For the slowest-growing population, the relative abundance of cells in fusiform shapes was 96.2% compared to 90.0% for the fastest-growing population. For the oval shape, there was an increase of 1.6% between the slowest and fastest-growing populations. Regarding the cell shape diversity, the Shannon-Wiener diversity index went up with growth rate, as slower-growing *Phaeodactylum* populations ($\mu$ = 0.1-0.5 $d^{-1}$) mainly consist of cells in their fusiform, while in the faster-growing populations ($\mu$ = 0.6-1.0 $d^{-1}$) more cells shift to their triradiate and oval shapes, which led to a higher diversity index value of 0.19 along the gradient (**Fig. 4**). Overall, we observed an upward exponential trend in cell shape diversity ($R^2$ = 0.63, $p$ = 0.012) along the growth rate gradient. During the assessment of the light micrographs, we observed no cells in their rare cruciform shape.

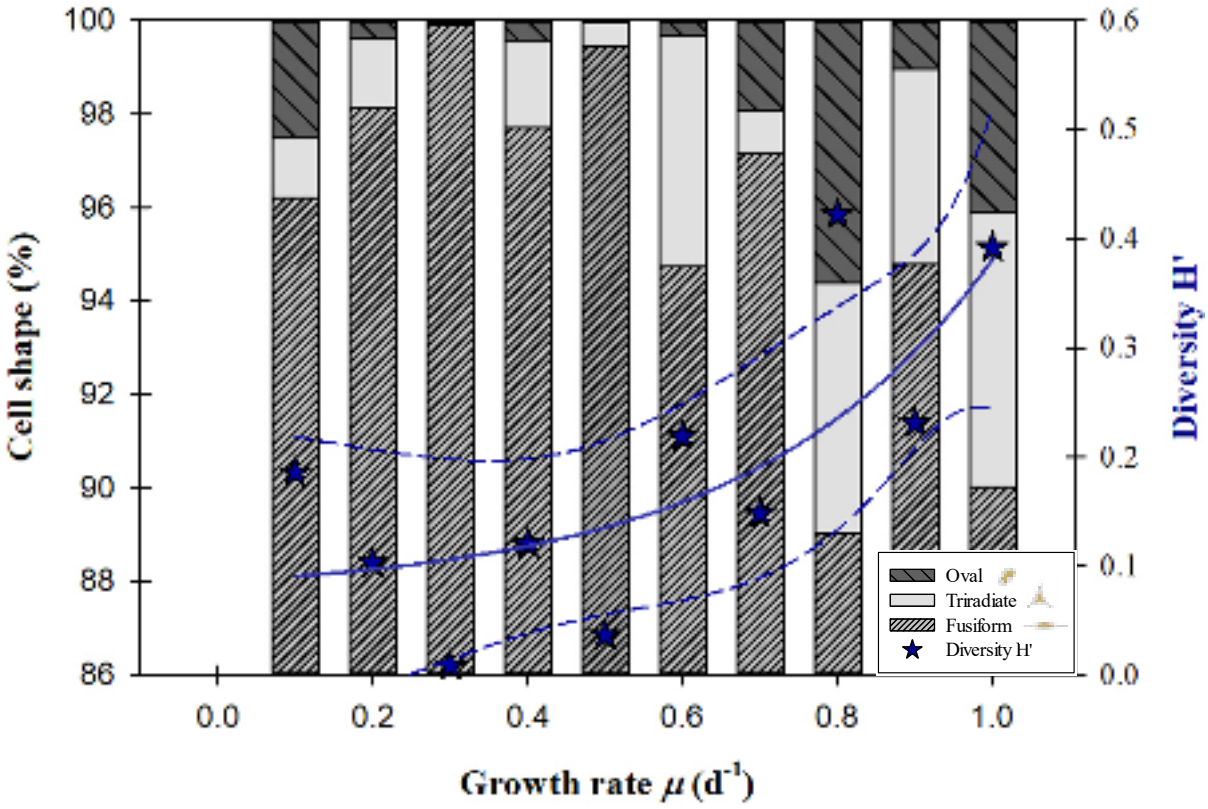


**Figure 4: Relative distribution of three present cell shapes in the differently growing *Phaeodactylum* populations and their corresponding Shannon-Wiener diversity index (*H'*) values plotted in blue.** We included three drawings of the corresponding shapes retrieved from www.biorender.com in the legend. We fitted through the *H'* values an exponential growth regression model combined with 95 % short-dashed confidence intervals along the growth rate gradient.

# Discussion

Here, we assessed whether faster-growing cells of one genotype of the marine diatom *Phaeodactylum* had elemental ratios approaching the Redfield ratio, as predicted by Goldman *et al.* (1979), as well as lower intercellular trait variability as predicted by Hillebrand *et al.* (2013). We showed that our hypotheses of downward trends (in P-limited systems) with increasing growth rate in N/P indeed approached the Redfield ratio,

as well as most of the cell-to-cell variability were met, and also hold true within populations of a single algal genotype.

## Linking algal growth rates to N/P stoichiometry

We aimed to link several functional traits - elemental stoichiometry, cell size and shape and cellular pigment content and complexity - to growth rate. For the bulk elemental stoichiometric ratios (C/N/P), we observed downward trends in all ratios with elevated $\mu$. We found that the faster-growing algal populations contained more phosphorus (P), as predicted by the growth-rate-hypothesis for P-limited systems (Sterner & Elser, 2002; Flynn *et al.*, 2010). These results agree with previous chemostat experiments assessing three algal species from distinct marine taxa, a marine cyanobacterium *Synechococcus* sp. and one freshwater green alga *Selenastrum minutum* (Goldman *et al.*, 1979; Elrifi & Turpin, 1985; Garcia *et al.*, 2016). In the study by Maat *et al.* (2014), this drop in stoichiometric ratios were also observed in the fast-growing chemostat populations ($\mu$ = 0.70 $d^{-1}$) compared to the slow-growing chemostat ones ($\mu$ = 0.23 $d^{-1}$) for the marine picoplankter *Micromonas pusilla*.

The measured stoichiometric elemental ratios of the model diatom *Phaeodactylum* were all approaching the Redfield ratio at high growth rates. This is in agreement with the work by Goldman *et al.* (1979), Loladze and Elser (2011) and Hillebrand *et al.* (2013), which overall hypothesized that when species grow faster, the ratios of their particulate elemental composition is converging to the Redfield ratio as global attractor, as a N/P of ~16 is intrinsically rooted in growth physiology (Loladze & Elser, 2011). This specific ratio is linked to the average N- and P-pools of all major macromolecular compounds of a phytoplanktonic cell growing at its maximum rate under optimal growth conditions, when there is a balance of these compounds (Loladze & Elser, 2011). Moreover, a comparable cellular convergence in N/P ratio to Redfield with higher growth rate was also observed in e.g., Rhee (1978) and Garcia *et al.* (2016). We linked for the first-time intercellular trait variation to elemental stoichiometric compositions.

## Linking algal growth rates to intercellular trait variability

Our results show lower intercellular variability in traits when the model diatom *Phaeodactylum* grew faster. To determine the within-culture intercellular variability, we estimated three functional traits, pigment content, cell size and cellular granularity using flow cytometry and one, cell shape, using microscopy. For the traits analysed by flow cytometry, we indeed observed decreasing variability with growth rate for pigment content and cell size. Thus, as was the case for the observations between cultures, variation also decreases within cultures. In contrast, and somewhat unexpectedly, the pattern with cell shape was opposite, with higher diversity at higher growth rates.

An increasing number of studies have investigated intraspecific variability between and within genotypes of one species (e.g., Kremp *et al.*, 2012; Garcia *et al.*, 2016; Brandenburg *et al.*, 2018; Groß *et al.*, 2021). Most studies observed both intraspecific and intrastrainal variability, but concentrated on solely bulk measurements on monogenetic population-level. The big difference with our study is that we combined similar bulk measurements with measurements on intercellular level to corroborate potential trends between intercellular variability and growth rate.

There are several potential explanations for enhanced intercellular trait variability at lower growth rates. *First*, at high growth rates everything is optimal, as otherwise the cells could not be growing so fast, and as a result all the cells should be similar in their biochemical make-up. *Second*, potentially, as a result of the very fast growth, cells are typically dividing synchronously, which would make them more similar to each other than cells that have lower division rates and co-occur in different stages of the division process (Massie *et al.*, 2010). *Third*, under more favourable growth conditions (higher growth rate) there is a low demand for plasticity, for example in nutrient acquisition strategies, leading to lower intercellular variability in those traits (e.g., West-Eberhard, 2003; Collins & Schaum, 2021). *Fourth*, the intercellular

variability could have been caused by the culture regime, as chemostats also simultaneously control the mortality rate/residence time and hence determine the duration individual cells spend in the culture vessels. High dilution rates (high growth rates) create a population of young individuals, most likely with similar traits. Lower dilution rates will also allow older individuals to remain in the vessels. If trait variation is related to age variation then we would expect the variation to indeed decrease with increasing growth (dilution) rates. However, the recent study by Groß *et al.* (2021) reported lowered intercellular trait variability with higher growth rate induced by global change related treatments of a single genotype diatom kept in batch, when residence time does not play a role. *Fifth*, part of the increased intercellular variability is a result of enhanced stickiness of the cells caused by amplified C-excretion (at lower growth rates) to buffer nonoptimal internal elemental compositions (e.g., Engel, 2000; Klein *et al.*, 2011). Cells sticking to each other would increase both overall cell size and cellular pigment content as well as cellular granularity, which we observed in the cytograms (i.e., the tails in the upper right corner), and which could be linked to slightly elevated organic material fluxes in the oceans (e.g., Fowler & Knauer, 1986). *Last*, the results might be snap shots of phenotypic selection towards optimal phenotypes in the slower-growing populations as well, i.e., the slower-growing populations might have been in a transitional state towards specific optimality at the time of the harvest eventually obtained via competitive exclusion. Despite that we used a monoculture with one genotype, the phenotypic trait values are obviously not all exactly the same, e.g., there is always some spread in cell size among cells, due to distinct phases of cell division and slight variation in environmental cues. In other words, there was likely some phenotypic variation present in the chemostat inoculum that got sorted via intraspecific competition among diverse phenotypes. The latter could be tested by running the same experiment longer. Irrespective of the explanation, the pattern is clear, and could have repercussions on ecosystem functioning (see below).

The higher diversity in shape at higher growth rates is difficult to explain. Essentially the fusiform shape is being replaced by the two other forms in more or less equal amounts at higher growth rates, which increases the shape diversity via *morphotype sorting*. One of the biggest consequences of shape change is the change in surface area-to-volume ratio (SA/V). Cells in their triradiate shape have a higher SA/V (Bartual *et al.*, 2008) and hence an increase in nutrient uptake efficiency (Thingstad *et al.*, 2005; Litchman *et al.*, 2007) and oval-shaped cells a lower SA/V with a reduced uptake efficiency. Noteworthy, larger cells with more vacuoles can store more macromolecular compounds (e.g., Thingstad *et al.*, 2005). Thus, when *Phaeodactylum* is growing slower, fusiform cells with an intermediate SA/V dominate, potentially in a trade-off of balancing nutrient uptake and storage, as these cells also had the highest granularity, indicating that they were storing large amounts of especially carbon in the cells. Song *et al.* (2020) determined that fusiform-shaped cells are rich in C-rich lipids and carbohydrates (i.e., cellular building blocks partly determining cell size), while oval-shaped cells were rich in N-rich proteins and pigments (i.e., linked to the autofluorescent pigment signal). Thus, the differences in shapes might be the consequences of the need for storing different compounds (carbon in the nutrient-limited slow growers and nitrogen and phosphorus in the fast-growers). We showed that the populations which were dominated by fusiform-shaped cells were also C-richer. Moreover, these populations had higher cellular granularity values. All in all, it seems that in our study the dominant growth strategy under less optimal growth conditions was luxury consumption combined with storage, which as a consequence of these demands led to fusiform-shaped cells. This strategy led to fuller cell compartments with macromolecular (carbon) compounds and hence elevated mean cellular granularity levels under less favourable growth conditions that led to reduced growth rates. At higher growth rates, the storage demands were lower and different, and hence the pressure of fusiform shape relaxed.

## Ecological implications

In the ecological literature, the diversity of prey availability has been linked to the stability of ecosystems, efficiency of food chains and much more. Especially phytoplankton diversity has received a lot of attention (Hillebrand *et al.*, 2009; Striebel *et al.*, 2016), with strong foci on species diversity. Here, we show that even within genetically identical strains of one phytoplankton species diversity is possible, and this diversity is predictably dependent on the growth rate of the cells. Thus, depending on the growth rate of primary producers, the present prey offers different quality to their grazers. This might have consequences. For instance, the study by Malzahn and Boersma (2012) tested the influence of these C/N/P ratios on copepod's ontogeny. When they fed the calanoid copepod *Acartia tonsa* with P-rich and P-poor prey items of the same unicellular algal strain, they observed that its growth was delayed, when the copepod was feeding on high C/P food. Thus, a faster-growing algal population could lead to elevated growth rates of higher trophic levels as they present a higher quality food (e.g., Malzahn *et al.*, 2007). At the same time, the higher variation (phenotypic diversity) at lower growth rates might create windows of opportunity for a more diverse set of grazers, thus potentially lower growth in the primary producers would be able to sustain a more diverse grazer community. Another potential ecological implication of differently growing algae is their effect on production of their corresponding viruses (Bratbak *et al.*, 1993; Clasen & Elser, 2007; Maat *et al.*, 2014). Viral production is sensitive to both phosphorus and nitrogen limitation, as P or N-depleted viral hosts lead to lowered production in viruses (Maat & Brussaard, 2016). Thus, viruses would proliferate better, when infecting fast-growing host cells, and the rate at which specific algal populations are growing can also control the speed and impact of the top-down regulation by viruses in the field (Bratbak *et al.*, 1993; Brussaard *et al.*, 1995).

In summary, in non-genetic within species diversity, related to the growing conditions of the phytoplankton, constrained trait variation combined with convergence towards a singular value in the direction of the Redfield ratio is real. It has the potential to affect higher trophic

levels, both by changing the overall availability of nutrients as well as affecting the variation of potentially available prey.

# Acknowledgements

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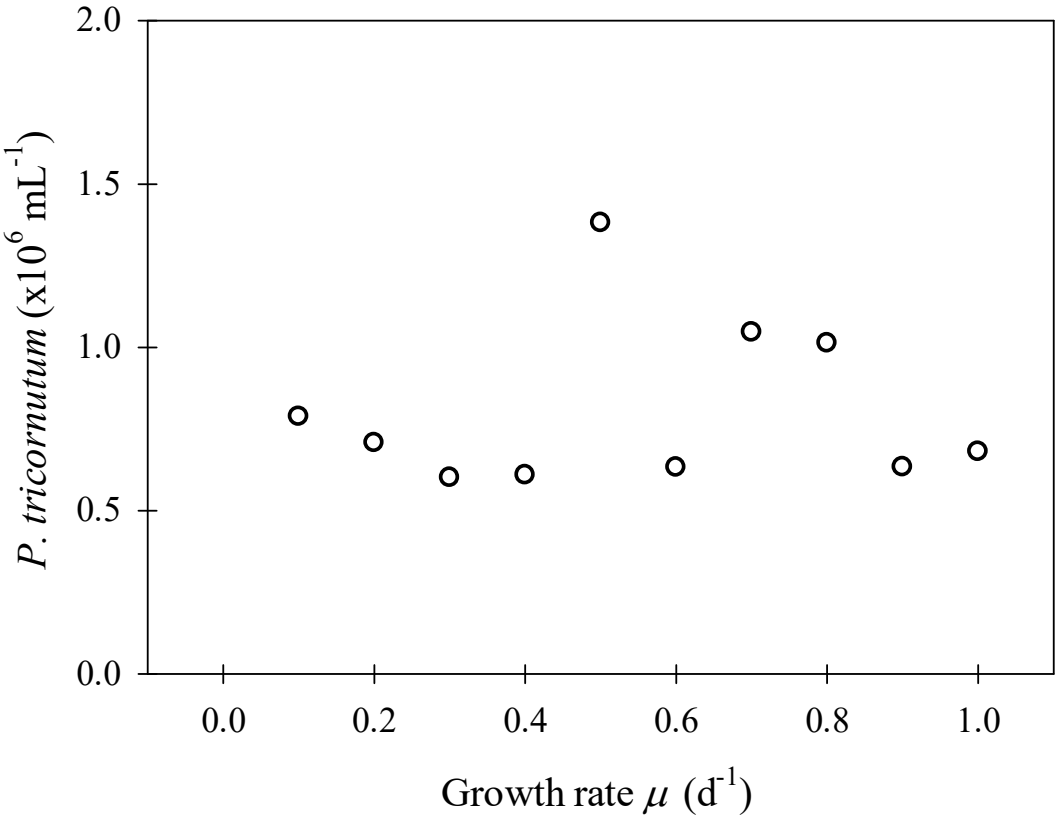
# Supporting information

## Tables

**S1 Table: Dissolved concentrations ($\mu$mol $L^{-1}$) and elemental ratios (molar) in each chemostat stated with their selected dilution rate (D; $d^{-1}$) of the measured nutrients nitrate ($NO_3^-$), nitrite ($NO_2^-$) and phosphate ($PO_4^{3-}$).** For determining the elemental ratio with N, we combined the nitrate and nitrite pools.

| Supply N/P | Chemostat (Number) | D | $NO_3^-$ | $NO_2^-$ | $PO_4^{3-}$ | N/P |
|---|---|---|---|---|---|---|
| **24.3** | **1** | **0.1** | 241.52 | 43.78 | 1.88 | 151.63 |
| **24.3** | **2** | **0.2** | 402.55 | 13.43 | 1.69 | 246.58 |
| **24.3** | **3** | **0.3** | 349.31 | 30.19 | 1.72 | 220.12 |
| **24.3** | **4** | **0.4** | 345.11 | 29.92 | 1.75 | 214.92 |
| **24.3** | **5** | **0.5** | 350.44 | 6.42 | 1.59 | 225.08 |
| **24.3** | **6** | **0.6** | 378.67 | 6.74 | 1.84 | 209.86 |
| **24.3** | **7** | **0.7** | 359.64 | 7.33 | 1.52 | 242.22 |
| **24.3** | **8** | **0.8** | 460.00 | 4.30 | 1.36 | 340.90 |
| **24.3** | **9** | **0.9** | 403.75 | 4.85 | 1.74 | 234.89 |
| **24.3** | **10** | **1.0** | 388.63 | 6.01 | 1.63 | 242.70 |

## Figures




**S1 Figure:** Cell density of the 10 *Phaeodactylum tricornutum* populations at the harvest day of the chemostats.

# Chapter 5: General Discussion & Outlook

# Part I: General Discussion

## 5.1: Discussion

Phytoplankton form a major link in planktonic food webs, contributing massively to the world's oxygen production, biogeochemical cycling and the transfer of organic matter and energy through the aquatic food web (Falkowski *et al.*, 1998; Field *et al.*, 1998). However, despite the importance of planktonic organisms, several smaller and larger gaps in our knowledge still exist, which I aim to fill with this thesis. First and foremost, the interactions between different components of the food web, especially in the highly dynamic spring blooms, are, despite years of research (Sommer *et al.*, 1986; Löder *et al.*, 2011), somewhat sketchy still. Especially the relative importance of bottom-up versus top-down control of microbes is unresolved. Hence, to set the scene, I, first of all, investigated these in a spring bloom situation at Helgoland Roads (**Chapter 2**). I observed that grazing is a major driver of bacterioplankton and phytoplankton dynamics, especially in the second phase of the bloom, but that the grazing pressure on different potential zooplankton resources is highly variable. Based on this, I hypothesized that the growing conditions of the algae might be the main source of these differences. To investigate this, I first catalogued the wide variety of available media for algal growth in the literature (**Chapter 3**), and followed this up with a study investigating the importance of different growth conditions (in this case, algal growth rate) on algal traits, and their variability (**Chapter 4**). In this general discussion of the thesis, I connect up the different aspects of this study, entering a few new data, where I investigated the impact of the different algal growth conditions on a few selected grazers, showing indeed that, depending on the growth rate of algae their quality as food for grazers varies.

Planktonic consumers can graze on bacterioplankton and phytoplankton, thereby affecting total standing stocks and the corresponding ecological functions performed by their prey. In **Chapter 2**, I investigated the role of nano- and micro-sized (2-20 and 20-200 $\mu$m, respectively) zooplankton in shaping spring bacterioplankton and phytoplankton blooms, following Löder *et al.* (2011). I determined in **Chapter 2** that the top-down pressure these combined consumer groups exert on their prey can shape those blooms to the same extent as bottom-up pressures, such as resource availability. I, furthermore, showed that zooplankton are able to select both between bacterio- and phytoplankton prey groups, as well as within prey groups, and that zooplankton can actively select for bacterioplankton prey to satisfy their nutritional demands. In the current literature, the consensus is that especially bacterioplankton blooms are primarily algal-derived substrate-driven (Teeling *et al.*, 2012), and that predation does not play an important role. However, I showed that the grazing pressure exerted by zooplankton on bacterioplankton is high enough to actually create a substantial pressure on the population, with the potential to actually shape spring bacterioplankton and phytoplankton blooms. Even though I could show that the selectivity of the zooplankton on the different components of their potential food was strong and variable, the factors that drive this selectivity are much more diffuse. One potential factor that influences the quality of algae as food for zooplankton is the nutrient content of the algae.

As indicated in the introduction, one of the central dogmata of the fate of elements in the world's oceans is that they are typically found in a certain ratio, the Redfield ratio of 106/16/1 (C/N/P molar). Indeed, for the open oceans, these fixed ratios seem to be well described, variation may be caused by differences in nutrient inputs (e.g., Copin-Montegut & Copin-Montegut, 1983; Laws *et al.*, 1984; Takahashi *et al.*, 1985), but should be regulated back to a N/P ratio of 16 by changes in nitrification or denitrification (Weber & Deutsch, 2012). However, the central proposition of one ratio to rule all has received quite some criticism in recent years, and it is now well established that variation in both dissolved nutrients and the

composition of the seston may be higher than previously described (Sterner *et al.*, 2008; Martiny *et al.*, 2013; Frigstad *et al.*, 2014). One of the potential explanations for these seemingly conflicting results is the finding that the nutrient ratio found in algae is actually related to the growth rate of the algae. At high, maximal, growth rates, many algae show ratios of N/P in their tissue, which are very close to Redfield – with a nitrogen-to-phosphorus ratio (N/P) of around 16 (Redfield, 1958; Goldman *et al.*, 1979; Sterner & Elser, 2002; Hillebrand *et al.*, 2013). In other words, fast-growing phytoplankton populations have RR-like elemental composition values, where slower-growing ones do not. Thus, since phytoplankton goes through a range of different growth rates also during a bloom, I predicted that they should also have different nutrient concentrations during these phases, with potential repercussion for zooplankton selectivity and growth. However, before I could answer this question, it was necessary to investigate whether the results obtained before also held for genetically identical organisms such as those dominating a phytoplankton bloom.

## 5.2: Linking cellular elemental compositions to phytoplanktonic growth rate

A meta-analysis with 43 data sets of differently growing mono- and polycultures of phytoplankton indicated a lowering in variation in elemental composition and a convergence towards Redfield-like values (N/P = 16) with higher growth rates (Hillebrand *et al.*, 2013). From this analysis, I investigated whether these findings also hold within genetically identical strains within one species. Indeed, I observed that using the diatom *Phaeodactylum tricornutum* and cryptophyte *Pyrenomonas helgolandii*, the ratio of N/P comes close to the Redfield ratio at high growth rates, whereas it deviates from RR at lower ones (**Fig. 5.2**). Further, as shown in **Chapter 4**, also the variation among single cells is higher at lower growth rates.

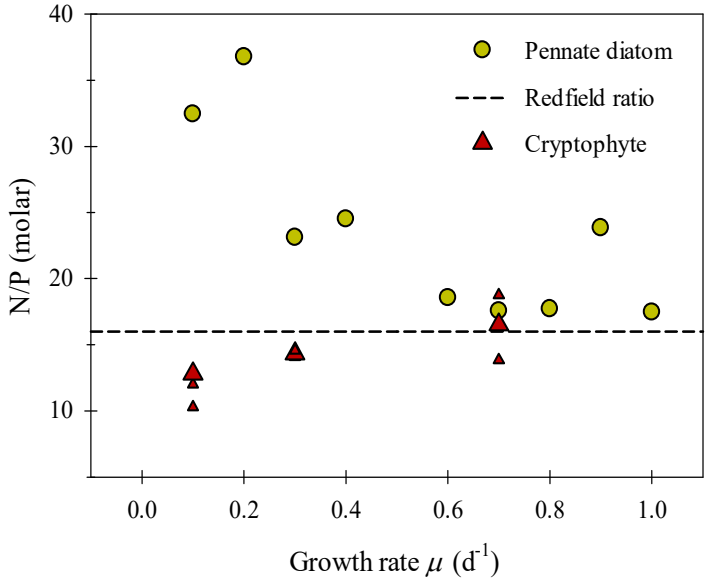



**Figure 5.2:** Funnel-shaped relation between cellular N/P ratios and growth rate observed in the diatom *Phaeodactylum tricornutum* (**Chapter 4**) and the cryptophyte *Pyrenomonas helgolandii* (unpublished data).

Thus, there are multiple ways (in terms of cellular content) to grow slowly, but only one way to grow fast. Hence, I observed lowered intercellular trait variability, which could have repercussions for higher trophic levels feeding on those algae. This optimal growth state of phytoplankton with high cellular growth rates, led me to question the general medium usage within the phytoplankton research field (**Chapter 3**). Whilst phytoplankton cells are non-homeostatic, their tissue composition follows the composition of external resources to a large extent, especially when growing at rates below the maximal growth rate (Sterner & Elser, 2002; Van De Waal & Boersma, 2012). In **Chapter 3**, I showed that the plethora of different nutrient recipes for different situations, species, laboratories, etc., is enormous. This means that comparing results of different studies is not trivial at all, as often the experiments were carried out with different nutrient conditions. Thus, to enhance comparability between studies, I advocate a much stricter use of a limited set of growth medium recipes.

I found that the set of media used in the literature is rather large, that growth rate has a very important role in determining the nutrient stoichiometry of algal cells (with higher variation and values further away from Redfield at lower growth rates), and that grazing plays an important role during spring blooms. The question now arises as to how to connect these findings. Based on these observations, I predict that different growing conditions of algae will yield different qualities of those algae as food for zooplankton, and as a consequence, it can be expected that zooplankton should grow differently on differently grown algae. To test the hypothesis presented in **Chapter 4**, whether slower-growing phytoplankton populations with higher intercellular trait variability that deviate from the Redfield ratio will lead to (i) less fit and (ii) more diverse consumers, and (iii) increased selective feeding behaviour, I performed an additional series of experiments. For this, I repeated the chemostat experiment presented in **Chapter 4** with a model cryptophyte *Pyrenomonas helgolandii*. I performed two major grazing experiments with the heterotrophic dinoflagellate *Oxyrrhis marina* to address the effects on grazer fitness and selective feeding behaviour, following Hantzsche and Boersma (2010). In these experiments, I observed an increased ingestion, and grazing preference for faster-growing (more P-rich) algal cells by *O. marina*, but no effect on predator growth rate (**Fig. 5.3**). Potentially, the range of C/P ratios in the experiment was such that they were around the optimal resource quality, and as a result I could not observe effects on growth. I corroborated that *O. marina* can actively select between algal subpopulations, growing at different rates and that *O. marina* has indeed weak homeostasis, whilst following its prey's elemental composition to some extent (Hantzsche & Boersma, 2010; Meunier *et al.*, 2012a).

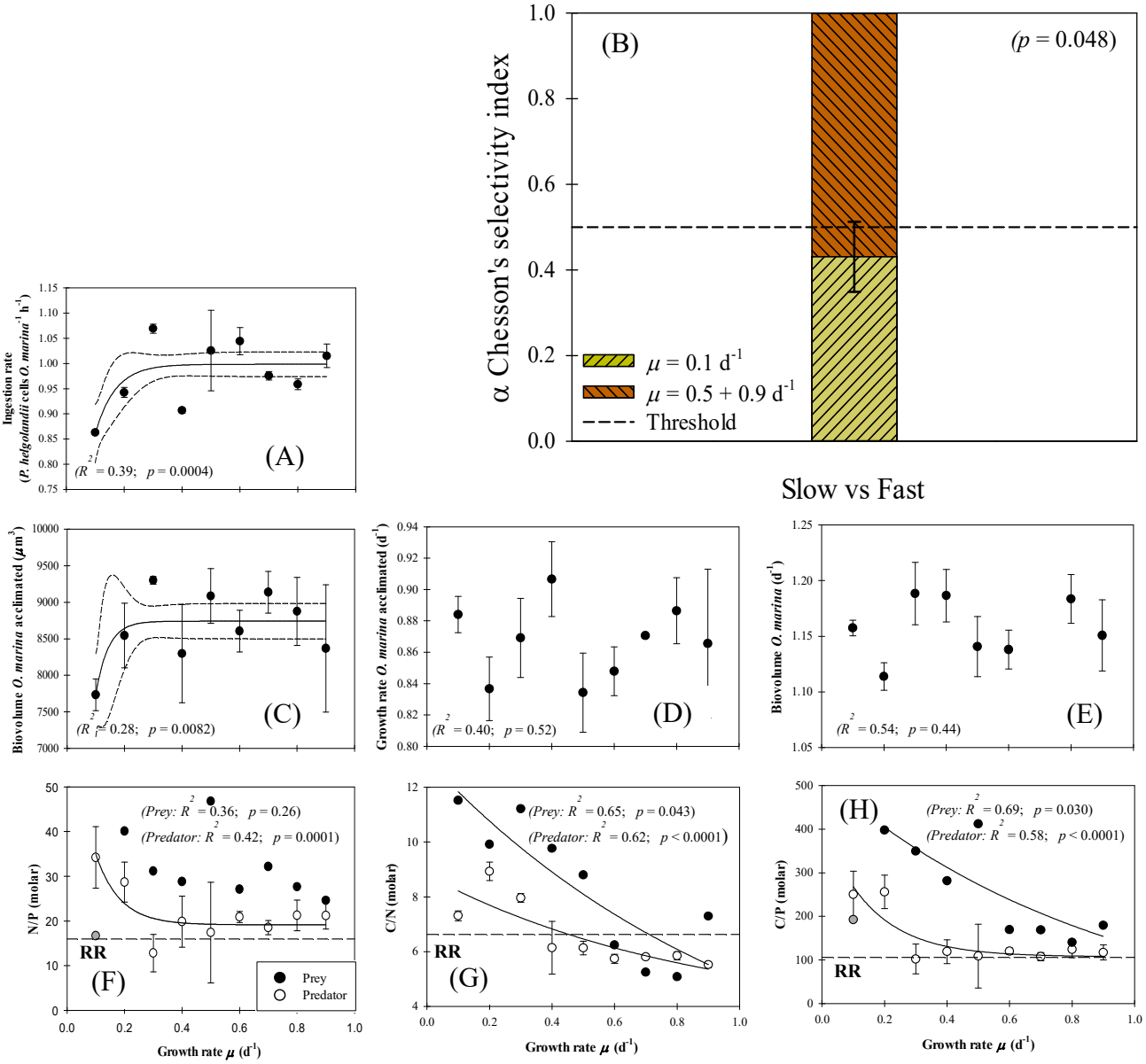



**Fig.5.3: Functional predator trait variation and selective feeding behaviour in response to algal prey growth rate.** In panels (A,C-E) the means of the trait values of *O. marina* fed with differently growing prey are indicated (mean ± SD, n = 4). In panels (F-H) the trait variations of the elemental compositions are shown for *O. marina* (open circle) and its prey *P. helgolandii* (solid circle) are shown after five feeding moments (for *O. marina*, mean ± SD, n = 3). The long-dashed line represents the Redfield ratio abbreviated RR. Significant trends are indicated with the solid 3-parameter exponential regression lines combined with dashed 95 % confidence interval. In panel (B), selectivity between equal amounts slow-growing (yellow) and fast-

growing *P. helgolandii* populations (orange). Selectivity was calculated as $\alpha$ Chesson's selectivity index (mean ± SD; n = 8). Dashed line represents no selectivity, values were significantly different from $\alpha = 0.5$, indicating selectivity. Significance was determined with a one sample t-test.

Further, to determine the direct impact of feeding on isogenic prey, growing at different rates, I performed an ontogeny experiment with an age-diverse copepod population, with the clear prediction that growth rate should be higher on faster growing cells, but at the same time that the diversity of life stages should be higher at the slower growing food, as the diversity of food qualities is highest there. I observed an increase in the growth rate of the age-diverse copepod subpopulation when fed with faster-growing algal cells, but found no impact between the copepod subpopulations due to phenotypic heterogeneity among the algal subpopulations (**Fig. 5.4**). A high functional diversity in a population and community may make it more productive and resistant to complete population or community collapse as a result of sudden pulse disturbances in abiotic conditions, such as heatwaves or storm events (Ptacnik *et al.*, 2008; Vallina *et al.*, 2017), albeit that a lower diversity will lead to a faster recovery after the disturbance (Baert *et al.*, 2016). Here, I detected no significant shifts due to the growth rate of the algal prey resulting from altered N/P supply ratios on two commonly used diversity indices the Shannon-Wiener diversity index and Pilou's evenness within an age-diverse copepod population (Weaver & Shannon, 1963).

Thus, in conclusion, I observed the reactions of grazers to the quality of the algae. *O. marina* selected for faster growers whereas *A. tonsa* developed faster on faster-growing cells. A question that arises from these results is whether these observations can also be made in the field.

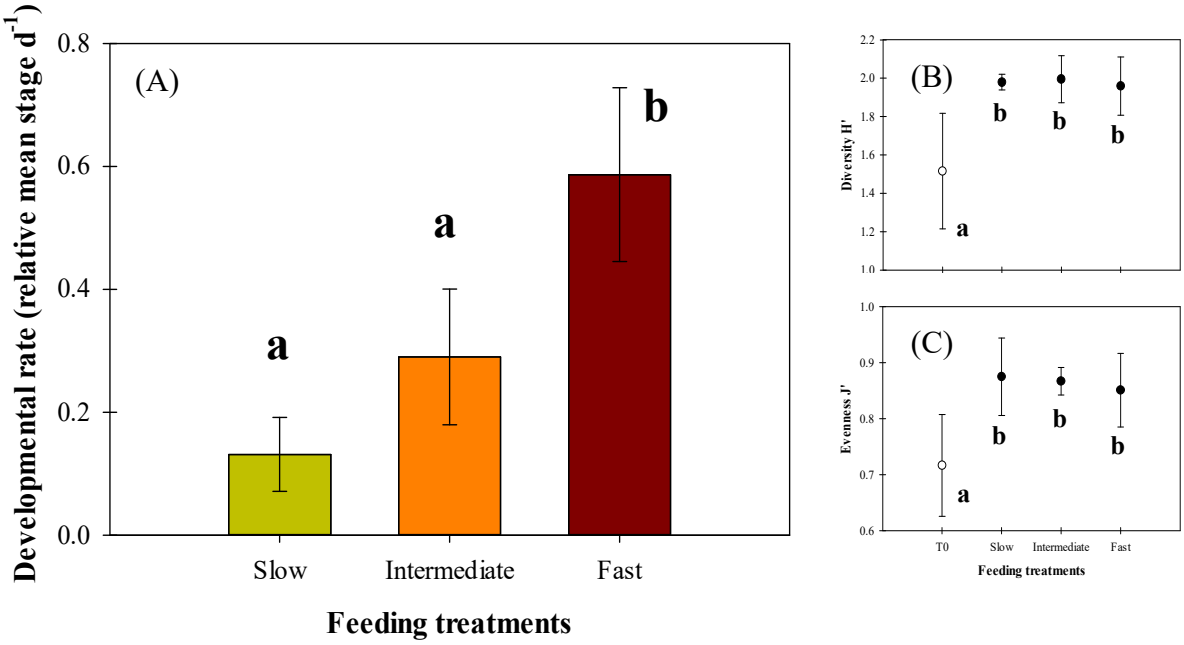



**Fig.5.4: Developmental rate of copepod stages and intraspecific stage diversity.** In (A) the developmental rate of the copepod stage assemblages when fed with slow (0.1 $d^{-1}$), intermediate (0.3 $d^{-1}$) and fast (0.7 $d^{-1}$) growing *P. helgolandii* cells after 5 days. In (B) the Shannon-Wiener diversity index (H'), and in (C) Pilou's evenness of the stage assemblages. The bars and symbols indicate the mean ± SD of 4 biological replicates. The open circles represent the initial state of the stage assemblage, and the solid circles the state after the feeding treatments. Different letters (a,b) indicate significant differences between treatments determined with one-way ANOVA's combined with for panel (A) a Bonferroni post-hoc test, and for panels (B) and (C) Tukey-HSD post-hoc tests.

## 5.3: Ecological consequences of differently growing algal prey for the food web

On seasonal, decadal, and centurial scales, there are trends and shifts in natural N/P loads impacting phytoplankton community structure and dynamics (e.g., Van Beusekom *et al.*, 2019), albeit that alterations in other abiotic parameters can co-influence community shifts (Mcquatters-Gollop *et al.*, 2007). In the field, a myriad of abiotic parameters can impact algal growth leading to modifications of ecosystem functioning. Namely, slower-growing species with higher trait plasticity (**Chapter 4**) using competitive strategies, such as storage-specialists and affinity-adapted species, contribute to stabilizing phytoplankton compositions. This happens by being less disturbed under rapidly changing environmental growth conditions (Sommer, 1984), and hence support ecosystem stability on a food web level (Lewandowska *et al.*, 2015), albeit co-dependent on phytoplankton attributes and growth strategies present within the community composition (Flöder & Hillebrand, 2012). All in all, the rate at which phytoplankton grow can have repercussion for the rest of the planktonic food web, and may even have consequences beyond the plankton level (Boersma *et al.*, 2008).

So, the question is, do we see the effects of algal growth rate on grazers also in the field? To assess whether interspecific or intraspecific variation in prey quality associated with different phytoplankton growth rates has a greater impact on the microzooplankton community's alpha diversity and/or the number of grazer species, as hypothesized in the thesis outline, data from **Chapters 2** and **4** can be combined (**Fig. 5.5**). The results indicate that there is no effect of growth rate of the phytoplankton *in situ* on the diversity of grazers present (data not shown). In contrast, the quality of the seston, in terms of N/P ratio of micro-sized (<200 $\mu$m) seston is correlated with the number of microzooplankton species observed, i.e., species richness (**Fig. 5.5A**; *S*; Exponential decay regression model, $P = 0.024$, $R = 0.53$) , but does not affect the community diversity (Exponential 3-parameter regression model; **Fig. 5.5B**: *H'*, $P = 0.39$, $R = 0.21$; not shown *J'*, $P = 0.27$, $R = 0.28$).

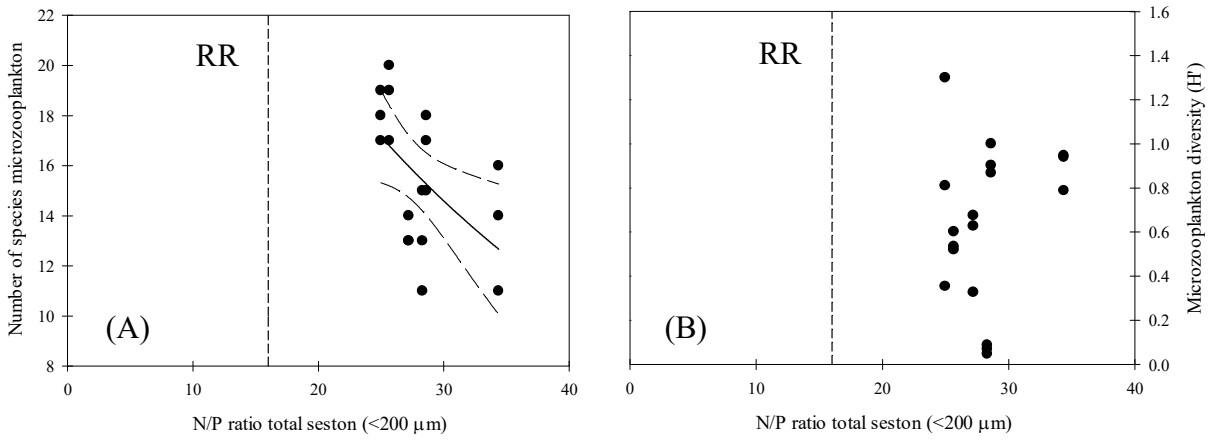



**Figure 5.5:** Correlation between N/P ratio of total seston and (A) species richness and (B) Shannon-Wiener index (*H'*). Dashed lines indicate confidence intervals.

In summary, I did find strong effects of grazing on phytoplankton and bacterioplankton, at the same time the growth rate of the phytoplankton affected the quality in terms of the ratio of N and P of several species of microalgae. Transferring this back to the field, yielded equivocal results, but despite the large scatter and the presence of many different algal species, I found indications that the same processes might also play a role in natural communities.

# Part II: Outlook

## 5.4: Outlook growth rate – from laboratory studies to the field

The German Bight (North Sea), my study area for this project, has experienced massive changes (~50-90%) of dissolved N and P concentrations between current trophic state of the system (2008-2010) compared to pre-industrial state (~1880; Kerimoglu *et al.*, 2018). These fluctuations were caused by intense eutrophication due to industrialization of the North Sea coast, and by following mitigation measures (Emeis *et al.*, 2015). It can be argued that the ongoing oligotrophication of the German Bight resulting from mitigation measures may influence the community structure as well as the trophic interactions within the planktonic food web in my study area. A recent trait-based analysis of temporal shifts in the phytoplankton community of Helgoland (1975-2018), showed a functional shift in community structure in the early 2000's (Di Pane *et al.*, 2022). Around this time, the phytoplankton community composition turned from slow-growing late-blooming organisms to fast-growing early-blooming organisms. This restructuring coincides with lowered dissolved P loads and warming. Di Pane *et al.* (2022) highlighted that these faster-growing early spring bloomers led to less fit copepod standing stocks and enhanced energy flow to the benthos.

In this thesis, I identified a tight dependency between phytoplankton growth rate and elemental composition, with a decreasing variability in C/P ratios at higher growth rates. I suggest that future studies should focus on linking phytoplankton growth rate with their elemental composition in natural conditions by, for example, conducting statistical analyses of long-term time series containing this data. Given that the relationship between growth rate and stoichiometry holds among taxa (Hillebrand et al. 2013), I would expect this pattern to be observed at the community level too. Furthermore, Hillebrand et al. 2013 identified that the identity of the limiting nutrient may influence the shape of the growth rate-stoichiometry

relationship. Analyses of long-term time series in regions such as the German Bight which underwent significant changes in dissolved nutrient concentrations may further help us unravel the consequences of these changes for phytoplankton physiology, and the implications for zooplankton consumers.

## 5.5: Outlook zooplankton grazing

To investigate the ecological consequences of intercellular trait variation related to growth rate in phytoplankton for herbivores, the results presented in **Section 5.3** call for follow-up studies. Future empirical work should focus on predator diversity related to feeding on algal subpopulations growing at different rates. To unravel the influence of changing dissolved nutrient concentrations on planktonic food webs, it is crucial to disentangle the underlying mechanisms of changes in higher trophic levels induced by food quality, in terms of elemental stoichiometry and morphological traits. Whilst the effects of prey stoichiometry on trophic interactions involving multispecies consumer assemblages have been addressed theoretically (Elser et al., 2012; Hall et al., 2004; Hall, 2009), Plum and Hillebrand (2019) conducted one of the few studies on the effects of altered stoichiometry on consumer species richness. These studies have suggested a potential strong effect on the coexistence of consumer species with different stoichiometric demands.

Here, I propose that future studies should test whether contrasting phytoplankton growth rates and stoichiometry alter multispecies consumer assemblages. To test this, natural microzooplankton could be sampled and fed with phytoplankton growing at different rates. By analysing the resulting taxonomic composition, we would obtain novel information on the influence of the growth rate-stoichiometry relationship on natural zooplankton assemblages.

Another promising approach is to pre-acclimate phytoplankton subpopulations cultured at different dilution (growth) rates, and subsequently mix those subpopulations with natural

zooplankton communities in dialysis bags placed into mesocosms or directly into the field (Stibor *et al.*, 2006), in addition to an earlier study with artificially obtained grazer communities (Plum & Hillebrand, 2019). The unified approaches of dialysis and dilution techniques enables the measurement of gross phytoplankton (and bacterioplankton) *in-situ* growth rates.

## 5.6: Outlook planktonic food web structuring and functioning

The results in **Chapter 2** raise the questions of how stable the observed trophic interactions are in other ecosystems and in a changing ocean. Yang *et al.* (2021) showed the immense difference in the trophodynamic role of microzooplankton between a spring and autumn bloom at the same sampling site with a more pronounced role during autumn bloom development. This suggests that the importance of grazing for the dynamics of bacterioplankton and phytoplankton observed in the spring study presented in Chapter 2, may be even stronger in autumn. This can be easily tested by repeating the same experiment – as described in **Chapter 2** – during an autumn bacterioplankton and phytoplankton bloom, which will likely lead to other species successions and interactions.

Next efforts should focus on predicting the impacts of future environmental conditions on microbial food web functioning and ecosystem stability (Lewandowska *et al.*, 2015). Indeed, recent studies suggest an increased flow of energy through the microbial loop in future planktonic food webs (Horn et al. 2020; Moreno et al. 2022). To unravel the consequences for overall food web functioning, and whether the flow of energy to higher trophic levels may be affected, experiments should be conducted to test the influence of different future scenarios. Mesocosm studies should test the stability of the trophic interactions observed in this thesis under different environmental conditions via a whole-planktonic-ecosystem approach (e.g., Horn *et al.*, 2020; Moreno *et al.*, 2022, for effects on food web structure). When focusing on

the microbial loop, these studies should separate the roles that nanozooplankton and microzooplankton play in grazing pressure on bacterioplankton and phytoplankton blooms. These organisms feed on a differently-sized prey, and at different rates, hence specific information on these aspects will substantially improve our understanding of how environmental change will influence energy flows within planktonic food webs.

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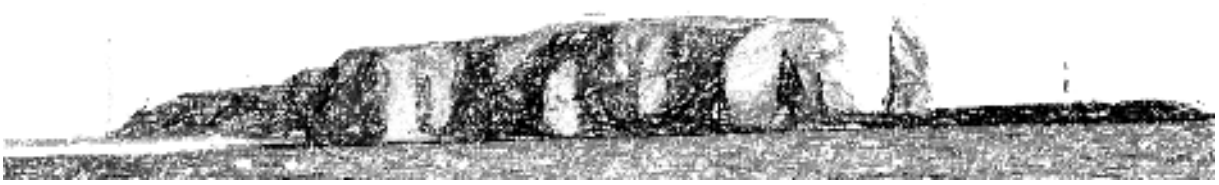

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