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Impacts of reduced nitrogen and phosphorus loads on coastal phytoplankton communities

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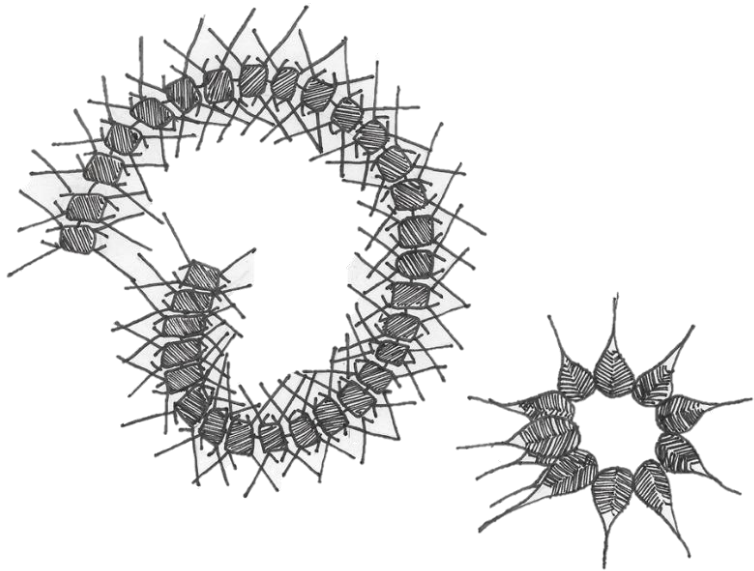
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Chapter 5

Competition for nutrients: seemingly neutral coexistence is stabilized by subtle niche differentiation



**Competition for nutrients:
seemingly neutral coexistence is stabilized by subtle niche
differentiation**

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Abstract

1. Niche-based theories and the neutral theory of biodiversity differ in their predictions of how natural communities respond to changes in nutrient availability. This is an issue of practical relevance, as many environments have experienced changes in nitrogen (N) and phosphorus (P) loads due to eutrophication and subsequent de-eutrophication efforts.
2. In the North Sea, disproportionate reduction of riverine P loads has led to a spatial gradient from P limitation of primary production in coastal waters to N limitation in offshore waters. To understand how this may impact community structure, chemostat experiments were conducted using a multispecies phytoplankton community sampled from the North Sea.
3. Results showed that picocyanobacteria (*Cyanobium* sp.) dominated the multispecies experiment under N-limitation, while picocyanobacteria and a non-motile nanoeukaryote (*Nannochloropsis* sp.) coexisted at equal abundances under P-limitation. Additional experiments using isolated monocultures confirmed that *Cyanobium* sp. depleted N to lower levels than *Nannochloropsis* sp., but that both species had nearly identical P requirements, suggesting neutral coexistence under P-limited conditions.
4. Pairwise competition experiments with the two isolates seemed to support the consistency of these results, but P limitation resulted in stable species coexistence irrespective of the initial conditions rather than the random drift of species abundances predicted by neutral theory.
5. Our results provide an interesting example where species were neutral competitors in one niche dimension, in terms of similar P requirements, thus seemingly supporting neutral theory. However, their competitive traits differed in subtle ways for other niche dimensions (i.e., the underwater light spectrum) ultimately leading to stable coexistence through niche differentiation.

Introduction

Eutrophication is a major environmental issue in many aquatic ecosystems (Nixon, 1995; Howarth et al., 1996; Carpenter et al., 1998). Increased N and P inputs often cause a decline in water quality, characterized by enhanced turbidity, increasing frequency and intensity of harmful algal blooms (Lancelot et al., 1987;

Anderson et al., 2002; Heisler et al., 2008) and the development of severe hypoxia (Diaz and Rosenberg, 2008; Conley et al., 2011; Breitburg et al., 2018). De-eutrophication attempts have only been partially successful, and the combined effect of eutrophication and subsequent de-eutrophication efforts have changed N and P loads in many waters (Grizzetti et al., 2012; Peñuelas et al., 2012; Chapter 2: Burson et al., 2016).

In coastal waters such as the North Sea, for example, increasing riverine N and P loads caused severe eutrophication in the mid and late 20th century (Pätsch and Radach, 1997; Philippart et al., 2000; Cadée and Hegeman, 2002; Lancelot et al., 2007). Subsequent nutrient reduction efforts resulted in effective P removal from domestic and industrial wastewater, but N loads have been lowered to a much lesser extent (Lenhart et al., 2010; Passy et al., 2013). In recent years, this unbalanced reduction of nutrient loads has led to a strong increase of the N:P ratio in nearshore waters, inducing a spatial gradient from P limitation of phytoplankton growth in the coastal region to N limitation in the central North Sea (Chapter 2: Burson et al., 2016). Understanding how these ongoing changes in N and P loads affect the productivity and species composition of marine ecosystems is a major challenge.

To predict how changes in nutrient loads will affect community composition, we may look to niche-based theories such as resource competition theory (Tilman, 1982; Grover, 1997; Brauer et al., 2012). By investigating the growth kinetics of a species when limited by a single resource we can determine the R^* value of that species for the resource, which is defined as the lowest possible environmental concentration of said resource at which the species can still thrive. Resource competition theory predicts that different species have different R^* values for a given resource, and that the species having the lowest R^* is the best competitor (Tilman, 1982). If there are trade-offs in the competitive abilities of species such that some species are better competitors for N and others are better competitors for P, then changes in environmental N:P ratios are expected to lead to predictable changes in species composition.

The neutral theory of biodiversity (Bell, 2000; Hubbell, 2001) offers an alternative explanation for species diversity which does not adhere to traditional niche differentiation. Instead, neutral theory contends that the high diversity is because all species within the same functional group are equivalent in their competitive ability. Random fluctuations in the demographic properties (birth,

death and migration rates) of species are in proportion to their relative abundances in the total community. This results in random ups and downs of population abundances, called ecological drift (Volkov et al., 2003; Hubbell, 2005; 2006; Shipley et al., 2006). Hence, neutral theory assumes that species abundances change by chance and not because of differences in competitive abilities (Hubbell, 2001; Etienne and Olff, 2004).

According to several recent studies, neutral coexistence might play an important role in multispecies phytoplankton communities (Vergnon et al., 2009; Chust et al., 2013; Segura et al., 2013; Mutshinda et al., 2016; Sakavara et al., 2018). Empirical evidence supporting these ideas is based on field data showing unexplained ('random') variation in the relative abundances of species in plankton communities (Chust et al., 2013; Mutshinda et al., 2016), or on clumpy distributions of species traits such as cell size (Vergnon et al., 2009; Segura et al., 2013). In the latter case, the idea is that species within these clumps have similar traits and, hence, their interactions are governed by neutral processes (Segura et al., 2013; Sakavara et al., 2018). However, while these results are promising, it is difficult to ascertain from field data whether the species concerned were indeed functionally equivalent. Some relevant environmental or biotic variables may have been overlooked, or species that are similar in some traits (e.g., size) may be differentiated along other niche dimensions. Controlled laboratory experiments with comprehensive investigations along multiple niche axes may provide more robust insight into the potential for neutral coexistence.

In this paper, we investigate whether shifts in N and P loads in aquatic ecosystems are likely to result in systematic changes in phytoplankton community composition attributed to niche differentiation or in random changes attributed to neutral competition. For this purpose, we added an inoculum of naturally occurring North Sea phytoplankton to laboratory chemostats to study shifts in species composition in response to different N and P loads. Next, we isolated the two most abundant species in these multi-species competition experiments, and determined their R^* values for N and P in monoculture experiments to assess whether there were trade-offs in their competitive abilities or if they were neutral competitors. Subsequently, we performed pairwise competition experiments in which the isolated species were inoculated at different initial relative abundances. If the two isolated species would be neutral competitors, then competition experiments in which the species start from

different initial conditions are unlikely to converge to the same species abundances. Conversely, if the two species show niche differentiation, pairwise competition experiments are likely to lead to the same stable species coexistence irrespective of the initial relative abundances of the species (although alternative stable states in community composition are also a possibility). Finally, we use the competition results to evaluate whether the observed species coexistence can be explained by functional equivalence of the species or whether niche differentiation should be invoked.

Materials and Methods

North Sea inoculum

Marine phytoplankton was collected from two locations in the North Sea during a research cruise in May 2012 aboard the Dutch research vessel *RV Pelagia* using a sampling rosette equipped with 24 Niskin bottles. Water was sampled from a nearshore station at 7 km from the Dutch coast (53°23'60" N, 5°9'0" E), and from an offshore station in the central North Sea (56°34'48" N, 2°10'12" E). At each station, water collected at 7 m depth was passed through an 80 µm mesh into a 20 L carboy to remove large zooplankton and debris, and then bubbled for 30 min with N₂ gas and 30 min with CO₂ to eliminate smaller grazers while providing inorganic carbon for phytoplankton photosynthesis. The carboys were kept at 4° C, until initiation of the chemostat experiments at the University of Amsterdam 2 days after the cruise ended.

Multispecies community experiments

The phytoplankton communities sampled from the North Sea were grown in laboratory experiments under either N-limited or P-limited conditions to investigate which species would become dominant under which nutrient limitation. The experiments were conducted in flat-walled chemostats (1.7 L working volume), with full control of light conditions, temperature, pCO₂ in the gas flow, and nutrient concentrations in the mineral medium (Huisman et al., 1999a; Passarge et al., 2006; Ji et al., 2017). Prior to the experiments, the water samples collected from the nearshore and offshore station were mixed in equal proportions, to ensure that the chemostats were inoculated with the same initial community composition. To initiate the experiments, two chemostats were both provided with 0.5 L of the mixed North Sea inoculum and filled up with mineral medium of 35 psu salinity. One of the chemostats received mineral medium with

a low N:P ratio of 4:1 (160 μM nitrate, 40 μM phosphate) to induce N limitation, whereas the other chemostat received a high N:P ratio of 60:1 (600 μM nitrate, 10 μM phosphate) to induce P limitation. All other nutrients in the mineral medium were provided at non-limiting concentrations (Chapter 3: Burson et al., 2018).

The front surfaces of the flat chemostat vessels were lit with a constant incident light intensity (I_{in}) of 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (PAR range, from 400–700 nm), provided by white fluorescent tubes (Philips PL-L 24W/840/4P, Philips Lighting, Eindhoven, The Netherlands). The chemostat vessels had an optical path length ('mixing depth') of 5 cm. Light transmission passing through the chemostats (I_{out}) was measured daily with a LI-COR LI-250 quantum photometer (LI-COR Biosciences, Lincoln, NE, USA) placed at ten evenly distributed positions at the back surface of the chemostat vessel.

Inorganic carbon was added as sodium bicarbonate (0.5 mM) in the mineral medium and as CO_2 mixed into filtered air, which was bubbled through the chemostats at a flow rate of 80 L hr^{-1} using Brooks® instrument pressure flow systems (Hartford, PA, USA). The partial pressure of CO_2 in the air flow was adjusted to maintain a pH of 8.2, which was checked daily with a SCHOTT pH meter (SCHOTT AG, Mainz, Germany). Bubbling of the chemostats further ensured homogeneous mixing of the phytoplankton community, while daily scraping with a magnetic stir bar minimized wall growth. Temperature was maintained at 16°C using cooling plates connected to a thermocryostat, and dilution rates of the chemostats were set at 0.15 day^{-1} . Samples for phytoplankton counts were taken three times per week. The multispecies experiments continued until the total biovolume and species composition of the phytoplankton community remained stable for at least 5 days.

Monoculture and competition experiments

We isolated the two species that became most dominant in the multispecies experiments with the North Sea inoculum using a serial dilution method in 96 well plates, diluting the cell abundances until only one cell per well was deposited. The rRNA gene of the isolated species was amplified for taxonomic identification, using PCR reactions of extracted genomic DNA with 16S rDNA primers for marine cyanobacteria (Nübel et al., 1997) and 18S rDNA primers for marine picoeukaryotes (Moon-Van der Staay et al., 2000) (Table S5.1). The PCR

products were purified and subsequently sequenced by long run Quick Shot sequencing on an Applied Biosystems 3730XL sequencer (Baseclear, Leiden, the Netherlands). BLAST (Altschul et al., 1990) was used to link the obtained sequences to species names.

Monoculture and competition experiments with the isolated species were conducted in N-limited and in P-limited chemostats using the same experimental conditions as described above. R^* values (*sensu* Tilman, 1982) for N and P were estimated as the steady-state concentrations of dissolved inorganic nitrogen (DIN) in the N-limited monocultures and of dissolved inorganic phosphorus (DIP) in the P-limited monocultures, respectively. Light absorption spectra of the monocultures were measured at a 0.4 nm resolution using an AMINCO DW-2000 double-beam spectrophotometer (Olis Inc, Bogart, GA, USA).

To test whether the outcome of competition depended on the initial conditions, the two species were inoculated in the competition experiments at an initial biovolume ratio of 50:1 and at an initial biovolume ratio of 1:50, resulting in a total of four competition experiments (2 nutrient levels x 2 initial conditions).

Phytoplankton and nutrient analysis

In the multispecies community experiments, small phytoplankton cells (diameter < 3 μm) were counted using an Accuri C6 flow cytometer (BD Biosciences, San Jose, California) equipped with a blue laser (488 nm) and red laser (640 nm). For this purpose, phytoplankton samples (4.5 mL) were preserved with 0.5 mL formaldehyde (18% v/v)-hexamine (10% w/v) solution in 5 mL cryogenic vials. These samples were placed in 4°C for 30 min, flash frozen in liquid nitrogen, and stored at -80°C until flow cytometry analysis. Larger phytoplankton cells (> 3 μm) were counted from samples (14 mL) preserved with 1 mL Lugol's iodine and stored in the dark at room temperature until analysis via an inverted microscope (DM IRB, Leica Microsystems, Wetzlar, Germany) using 1 mL gridded Sedgewick Rafter counting chambers. We counted the entire chamber or 200 cells per species depending on cell concentrations. Biovolumes of the phytoplankton were calculated from cellular dimensions and geometry according to Hillebrand et al. (1999).

In the monoculture and competition experiments, phytoplankton abundances were quantified as biovolume and as cell numbers using a CASY

TTC cell counter (OLS OMNI Life Science, Bremen), which distinguished between the two species based on their cell size.

Nutrient samples (15 mL) were gently filtered over a 0.22 μm polycarbonate filter into 20 mL polyethylene vials, and stored in the dark at -20°C until analysis. Nutrients were analyzed using standard colorimetric methods for nitrate and nitrite (Grasshoff et al., 1983), ammonium (Helder and De Vries, 1979), and dissolved inorganic phosphorus (DIP; Murphy and Riley, 1962). Dissolved inorganic nitrogen (DIN) was defined as the sum of nitrate, nitrite, and ammonium. Cellular nutrients contents were estimated from the total amount of nutrients consumed by the organisms (i.e., the difference between the dissolved inorganic nutrient concentration in the mineral medium supplied to the chemostat and in the chemostat vessel itself) and the measured cell numbers.

Results

Multispecies community experiments

The phytoplankton mixture sampled from the North Sea and used as inoculum for the multispecies experiments consisted of a species-rich community of nanoflagellates (31% of total biovolume), picoeukaryotes (30%) and diatoms (21%), with smaller contributions by dinoflagellates, non-motile nanoeukaryotes and picocyanobacteria. During the first few weeks, all species increased in biovolume, indicating that the experimental design provided suitable growth conditions for all species in this multispecies community (Fig. 5.1). After 20-60 days of growth in the N-limited chemostat, many species from the initial community were competitively displaced and in the end the N-limited chemostat was dominated by picocyanobacteria (83%) and a small but diverse group of diatoms (16%) (Fig. 5.1a, b). The P-limited chemostat also showed competitive exclusion of a variety of species, and converged to a stable coexistence of picocyanobacteria (48%) and non-motile nanoeukaryotes (48%) (Fig. 5.1c, d).

By means of serial dilution, we isolated the dominant picocyanobacterium from the N-limited chemostat and the non-motile nanoeukaryote from the P-limited chemostat. The 16S rRNA gene sequence of the picocyanobacterium belonged to the *Synechococcus/ Cyanobium* group, giving a 100% match with 16 strains of *Cyanobium* sp. and 2 *Synechococcus* sp. strains. The 18S rRNA gene sequence of the nanoeukaryote resulted in a 100% match with the eustigmatophyte *Nannochloropsis* sp. The sequences have been deposited in

GenBank and are available under accession numbers KP762160 and KP762161 for *Nannochloropsis* sp. and *Cyanobium* sp., respectively.

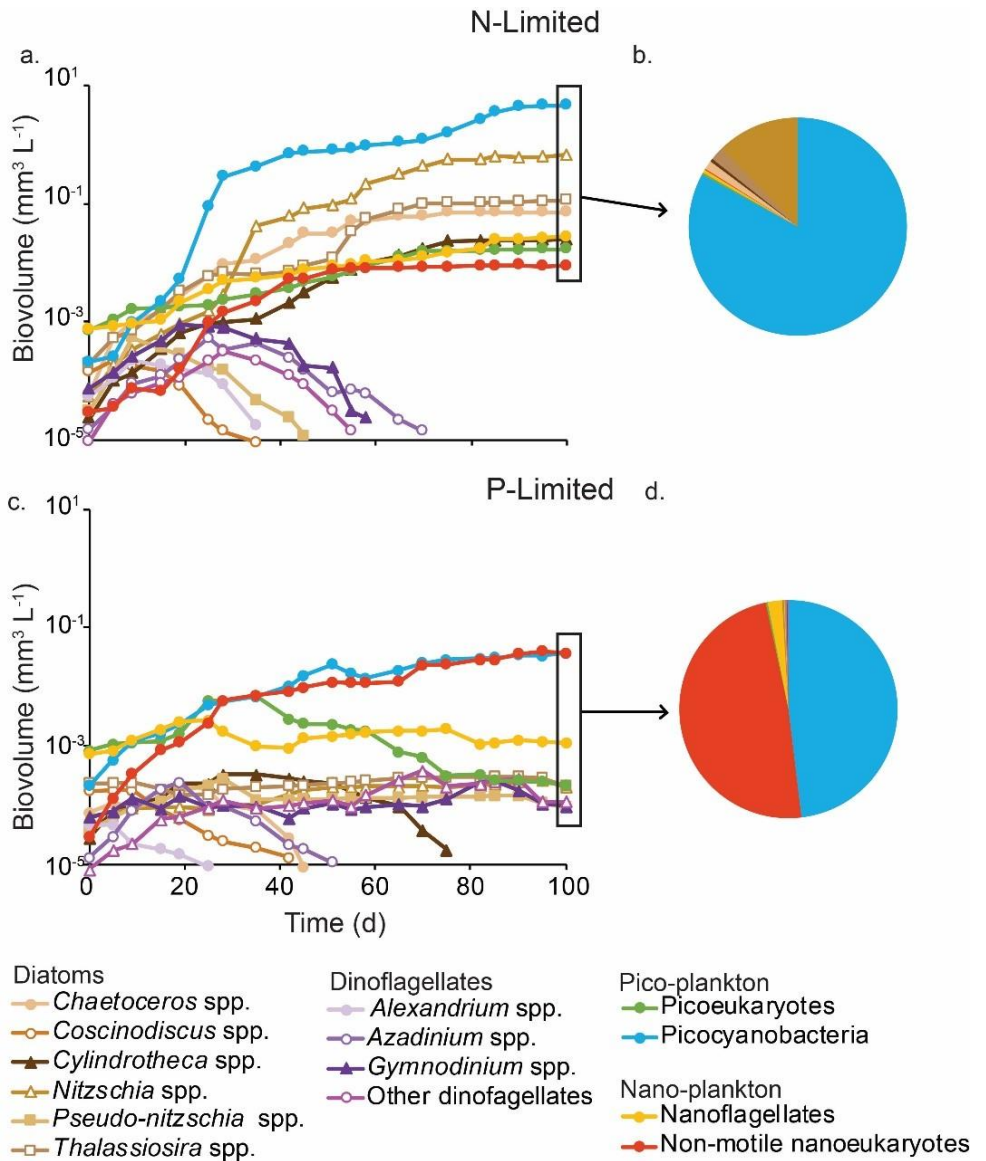


Figure 5.1 Multispecies community experiments with a phytoplankton mixture sampled from the North Sea. (a) Population dynamics and (b) final community composition in the N-limited chemostat, where mineral medium was supplied with a molar N:P ratio of 4:1. (c) Population dynamics and (d) final community composition in the P-limited chemostat, where mineral medium was supplied with a molar N:P ratio of 60:1.

Monoculture experiments

In monoculture experiments with the isolated *Nannochloropsis* sp. and *Cyanobium* sp., the phytoplankton populations increased (Fig. 5.2a) while light transmission through the cultures, DIN concentrations and DIP concentrations decreased until a steady state was reached after ~ 15 days (Fig. 5.2b-d). *Nannochloropsis* had lower cellular N and P contents than *Cyanobium* (Table 5.1), and reached a much higher total biovolume than *Cyanobium* under both N-limited and P-limited conditions (Fig. 5.2a). For both species, the steady-state biovolume was higher in the P-limited than in the N-limited monoculture. Accordingly, light transmission through the chemostats was reduced to $I_{out} \approx 20$ $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in the N-limited monocultures but to lower levels of $I_{out} \approx 10$ $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in the P-limited monocultures (Fig. 5.2b).

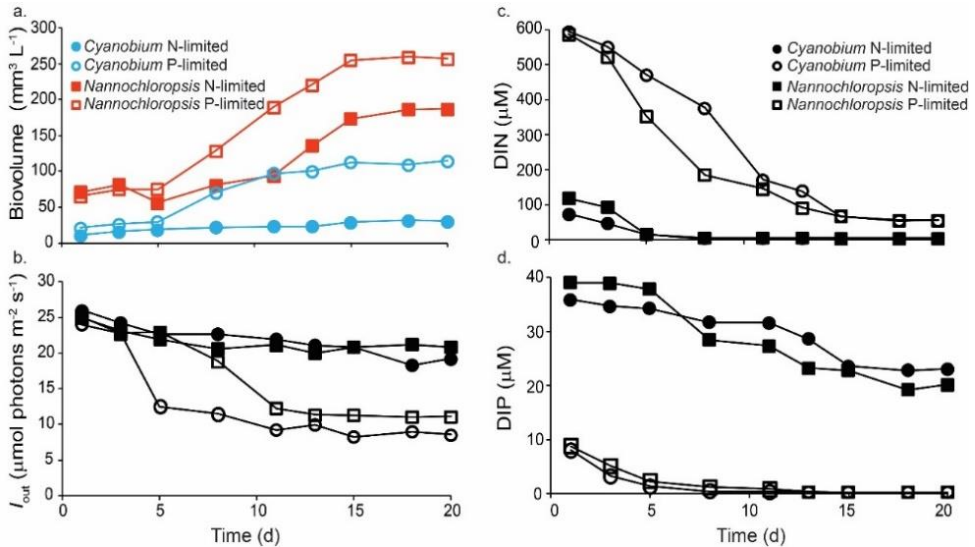


Figure 5.2 Monoculture experiments of *Nannochloropsis* and *Cyanobium* under N-limited and P-limited conditions. (a) Population dynamics of the species, (b) light transmission through the monocultures, (c) DIN concentrations, and (d) DIP concentrations in each of the four monoculture experiments.

In line with expectation, DIN concentrations were depleted to lower levels in the N-limited than in the P-limited monocultures (Fig. 5.2c). In the N-limited monocultures, *Cyanobium* depleted DIN to a lower steady-state concentration ($4.85 \mu\text{M}$) than *Nannochloropsis* ($5.43 \mu\text{M}$) (Table 1). Hence, *Cyanobium* had a lower R^* value for nitrogen, and is predicted to be a better competitor for nitrogen.

Conversely, DIP concentrations were depleted to lower levels in the P-limited than in the N-limited monocultures (Fig. 5.2d). *Cyanobium* and *Nannochloropsis* depleted DIP to similar concentrations of 0.23 and 0.22 μM , respectively (Table 5.1). Hence, the two species are predicted to be equal competitors for P.

Table 5.1 Nutrient requirements of *Cyanobium* and *Nannochloropsis* estimated from the monoculture experiments.

Parameter	<i>Cyanobium</i>	<i>Nannochloropsis</i>
Cell volume (μm^3)	0.47 ± 0.07	8.55 ± 1.37
Cellular N content		
- per biovolume ($\text{pmol } \mu\text{m}^{-3}$)	5.15 ± 0.00	0.85 ± 0.04
- per cell (pmol cell^{-1})	2.17 ± 0.17	6.12 ± 0.30
Cellular P content		
- per biovolume ($\text{pmol } \mu\text{m}^{-3}$)	0.087 ± 0.002	0.038 ± 0.00
- per cell (pmol cell^{-1})	0.049 ± 0.007	0.377 ± 0.008
R* value		
- for N (μM)	4.85 ± 0.14	5.43 ± 0.03
- for P (μM)	0.23 ± 0.02	0.22 ± 0.02

All values are based on the mean (\pm s.d.) of the last 5 time-points of the steady-state monocultures. Cellular N content and R* for N were determined in N-limited monocultures, and cellular P content and R* for P were determined in P-limited monocultures.

Pairwise competition experiments

In the two competition experiments under N-limited conditions, the time course of competition strongly depended on the initial conditions but the final outcome was the same (Fig. 5.3). When *Nannochloropsis* was inoculated with a 50x higher initial biovolume than *Cyanobium*, *Nannochloropsis* dominated the experiment for more than a month, reaching a very high peak abundance at day 26 (Fig. 5.3a). A few days after DIN was depleted below 5.4 μM , however, *Nannochloropsis* started to decline and in the end it was competitively excluded by *Cyanobium* (Fig. 5.3a, b). Conversely, when *Cyanobium* was inoculated with a 50x higher initial biovolume than *Nannochloropsis*, *Cyanobium* maintained its dominance throughout the experiment while *Nannochloropsis* was excluded (Fig. 5.3c, d).

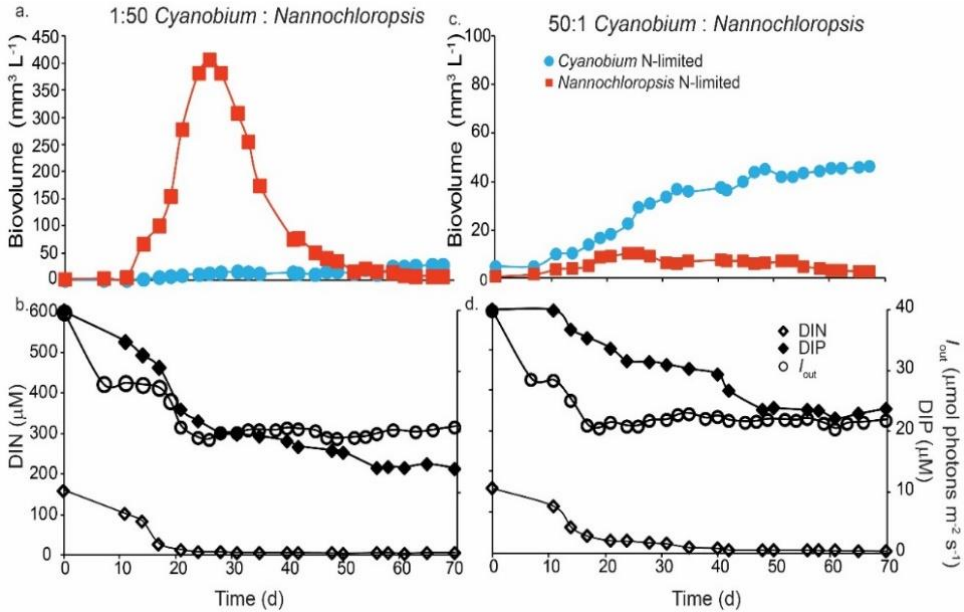


Figure 5.3 Competition experiments between *Nannochloropsis* and *Cyanobium* under N-limited conditions. (a, b) Time courses of (a) the competing species and (b) their resources when *Nannochloropsis* was inoculated with a 50x higher initial biovolume than *Cyanobium*. (c, d) Time courses of (c) the competing species and (d) their resources when *Cyanobium* was inoculated with a 50x higher initial biovolume than *Nannochloropsis*. Note the difference in scale between panels (a) and (c).

Under P-limited conditions, the time course of competition again depended on the initial conditions, but now both species coexisted throughout the experiments (Fig. 5.4). When *Nannochloropsis* was inoculated with a 50x higher initial biovolume than *Cyanobium*, *Nannochloropsis* again dominated the competition experiment during the first month and reached its peak abundance at day 24 (Fig. 5.4a). A few days after the DIP concentration was depleted, however, *Nannochloropsis* started to decline while *Cyanobium* increased. Once light transmission through the cultures had been brought down to $I_{out} \approx 12 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ and the DIN concentration had been reduced to $\sim 18 \mu\text{M}$, the *Nannochloropsis* and *Cyanobium* populations stabilized and the two species maintained a stable coexistence until the end of the experiment (Fig. 5.4a, b).

Conversely, when *Cyanobium* was inoculated with a 50x higher initial biovolume than *Nannochloropsis*, both species initially increased and then also converged to stable coexistence (Fig. 5.4c). The *Nannochloropsis* population stabilized from day 22 onwards when light transmission through the cultures had

been brought down to $I_{out} \approx 10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, while *Cyanobium* continued to increase for several weeks and reached a stable population from day 50 onwards when the DIN concentration had been reduced to $\sim 18 \mu\text{M}$ (Fig. 5.4c, d).

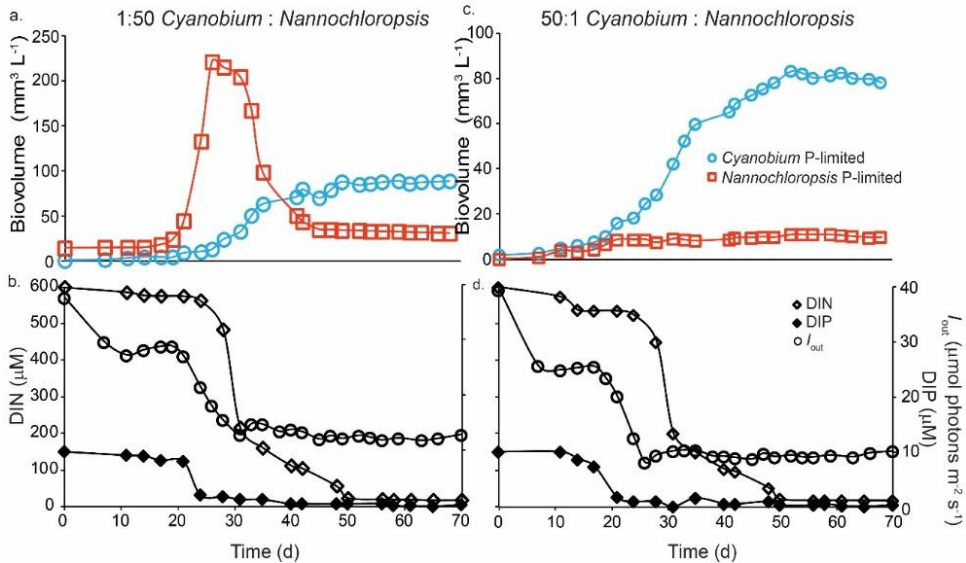


Figure 5.4 Competition experiments between *Nannochloropsis* and *Cyanobium* under P-limited conditions. (a,b) Time courses of (a) the competing species and (b) their resources when *Nannochloropsis* was inoculated with a 50x higher initial biovolume than *Cyanobium*. (c,d) Time courses of (c) the competing species and (d) their resources when *Cyanobium* was inoculated with a 50x higher initial biovolume than *Nannochloropsis*. Note the difference in scale between panels (a) and (c).

Discussion

Our results show that the outcomes of the pairwise competition experiments were independent of the initial conditions. Under N-limited conditions, the picocyanobacterium *Cyanobium* competitively displaced the nanoeukaryote *Nannochloropsis* (Fig. 5.3). Under P-limited conditions, *Cyanobium* and *Nannochloropsis* developed a stable coexistence irrespective of the initial abundances of the two species (Fig. 5.4). This indicates that these two species were not neutral competitors, but that their population dynamics were governed by differences in the competitive traits of the species.

Competitive replacement of *Nannochloropsis* by the picocyanobacterium *Cyanobium* under N-limited conditions is in line with the lower R^* value for N measured in the monoculture of *Cyanobium*. Interestingly, when

Nannochloropsis was inoculated at a higher relative abundance than *Cyanobium*, it developed an unexpectedly high abundance prior to its competitive replacement by *Cyanobium*. *Nannochloropsis* is known to accumulate high concentrations of polyunsaturated fatty acids (PUFA) under nutrient-limited conditions, which makes it a high-quality food source for zooplankton and fish larvae (Krienitz and Wirth, 2006) and a species of key interest in biotechnological applications (Hu and Gao, 2006; Pal et al., 2011). Its high fatty acid contents may explain why *Nannochloropsis* had low cellular N contents per unit biovolume in comparison to *Cyanobium* (Table 5.1), and hence, why *Nannochloropsis* could produce much higher biomass under N-limited conditions than *Cyanobium*. These results also demonstrate that the capacity of a species to produce high biomass does not necessarily provide a competitive advantage, because in the end *Nannochloropsis* lost the competition for N from *Cyanobium*.

Our monoculture experiments showed that *Cyanobium* and *Nannochloropsis* had very similar R^* values for P. Hence, according to resource competition theory, these two species should be (nearly) neutral competitors under P-limited conditions. In this case, neutral theory would apply, according to which the relative abundances of two species starting from very different initial conditions would drift more or less randomly in the competition experiments rather than converge to the same end state. Contrary to this expectation, we observed smooth rather than randomly fluctuating population dynamics under P-limited conditions. Moreover, the P-limited competition experiments ultimately led to similar outcomes, even though they started from very different initial conditions. These results clearly point at stable species coexistence with consistent final outcomes irrespective of the initial relative abundances of the species, rather than neutral coexistence where final abundances are randomly attained from ecological drift.

We note that DIN was reduced to relatively low concentrations of $\sim 18 \mu\text{M}$ in the P-limited competition experiments. Although this concentration remained above the low DIN concentrations in the N-limited competition experiments, it is sufficiently low to affect the growth rates of the species. Furthermore, light intensity was reduced to low levels of $10\text{--}12 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ in the P-limited chemostats (Fig. 5.4), which is considerably lower than in the N-limited chemostat (Fig. 5.3). Hence, in addition to the experimentally imposed P

limitation, N depletion and light limitation may have affected the competitive interactions of the species in the P-limited competition experiments, and therefore these experiments might have been co-limited by P, N and light rather than limited by P only. Application of the standard resource competition model for two essential nutrients (Tilman, 1982) predicts that, at the coexistence equilibrium, the DIN concentration should have been depleted to the R^* value for N of *Nannochloropsis*. This was not the case in our experiments. Hence, co-limitation by N and P (without co-limitation by light) is unlikely to explain species coexistence in the P-limited competition experiments. However, co-limitation by nutrients and light might offer an explanation for the stable coexistence of *Nannochloropsis* and *Cyanobium* observed in these experiments.

Theoretical and experimental studies have shown that phytoplankton species can exhibit stable coexistence by utilizing different wavelengths of the light spectrum (Stomp et al., 2004; 2007). We measured light absorption spectra of the two species to investigate this possibility. The absorption spectra of *Cyanobium* and *Nannochloropsis* largely overlap in the 400-470 nm range, but show distinct differences at longer wavelengths (Fig. 5.5). For instance, both species contain the ubiquitous pigment chlorophyll *a*, with which they absorb photons in the blue part (440 nm) and red part (680 nm) of the light spectrum, but the 680 nm peak of *Nannochloropsis* is much higher than that of *Cyanobium*. Furthermore, *Nannochloropsis* species are rich in carotenoids (Lubián et al., 2000) which are most likely responsible for the shoulder at 500 nm. Conversely, cyanobacteria use phycobilisomes containing stacks of accessory pigments, in this case of phycocyanin, which is responsible for the distinct absorption peak of *Cyanobium* in the orange region at 630 nm (Stomp et al., 2004; Haverkamp et al., 2009). Several studies have pointed out that the debate about niche differentiation versus neutrality can be reframed in terms of the relative importance of stabilizing mechanisms (niche differences) and fitness equivalence (neutrality) (Chesson, 2000; Leibold & McPeck, 2006; Adler et al., 2007). In theory, weak stabilizing forces are sufficient to enable stable coexistence of species with nearly equal fitnesses (Chesson, 2000). In the context of our experiments, this implies that subtle niche differentiation in the underwater light spectrum may have added a stabilizing factor to the otherwise neutral competition for P between the two species.

The pairwise competition experiments were consistent with our multispecies community experiments using North Sea phytoplankton. In both sets of experiments, picocyanobacteria became dominant under N-limited conditions, whereas picocyanobacteria coexisted with non-motile nanoeukaryotes under P-limited conditions. Furthermore, in another series of multispecies competition experiments with North Sea phytoplankton sampled during a different year, we found stable coexistence of picocyanobacteria, diatoms and green algae on a single limiting nutrient (Chapter 3: Burson et al., 2018). Although we did not determine R^* values of the species in those experiments, these taxa also differed in their photosynthetic pigments, and hence differences in light absorption spectra may explain their stable coexistence (Chapter 3: Burson et al., 2018). The overall consistency of this previous study and the current results further support our hypothesis that subtle niche differentiation in the light spectrum may provide a stabilizing mechanism for the coexistence of similar nutrient competitors.

Yet, the species composition obtained in our laboratory competition experiments deviated strongly from the natural phytoplankton community composition of the North Sea and other coastal waters. These differences in species composition might be dismissed as an experimental artifact owing to the highly artificial environments provided by laboratory experiments. However, the competitive replacement of large diatoms and dinoflagellates by small pico- and nanophytoplankton observed in our multispecies competition experiments is in line with the common expectation that small cells have a competitive advantage under nutrient-limited conditions (Raven, 1998; Irigoien et al., 2004; Edwards et al., 2011). Furthermore, one of the key differences between our lab experiments and natural waters is that we eliminated the zooplankton community. Hence, our findings also support the common idea that size-selective grazing by zooplankton plays an important role in the persistence of a broad size range of phytoplankton species in natural waters (McCauley and Briand, 1979; Steiner, 2003; Fuchs and Franks, 2010).

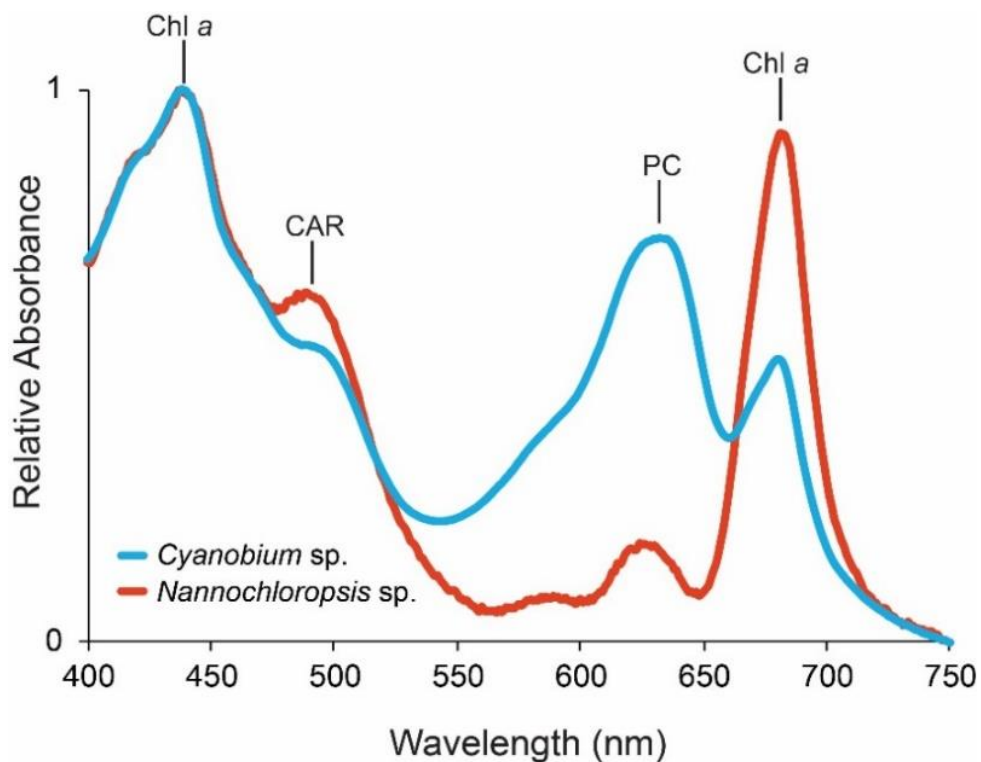


Figure 5.5 Light absorption spectra of *Nannochloropsis* and *Cyanobium*. Both species contain chlorophyll *a* (Chl *a*), absorbing at 440 nm and 680 nm. In addition, *Nannochloropsis* contains high contents of carotenoids (CAR) absorbing at 400-520 nm, whereas *Cyanobium* contains carotenoids and the phycobili-protein phycocyanin (PC) absorbing at 630 nm. The spectra were obtained under nutrient replete conditions.

It is important to emphasize that niche-based and neutral processes are not mutually exclusive (Leibold and McPeck, 2006; Adler et al., 2007). Instead, they represent the two extremes of a continuum of competitive interactions where sometimes niche differences (stabilizing mechanisms *sensu* Chesson, 2000) dominate and in other cases neutral coexistence (equalizing mechanisms) has the upper hand. Several recent studies have indicated that natural phytoplankton communities are characterized by a combination of niche differentiation and neutral processes (Vergnon et al., 2009; Chust et al., 2013; Segura et al., 2013; Mutshinda et al., 2016; Sakavara et al., 2018). For instance, analysis of long-term time series of diatoms and dinoflagellates in the western English Channel indicates that neutral coexistence is more likely within functional groups, whereas niche differentiation seems more important among functional groups (Mutshinda et al., 2016). In our study, subtle niche differences between

picocyanobacteria and nanoeukaryotes overruled their similar competitive ability for phosphorus, resulting in systematic rather than random changes among the two most abundant species in the multispecies experiments. Yet, we cannot exclude the possibility that neutral coexistence played a more prominent role for other species in our North Sea community. For instance, it might well be that the four diatom species that persisted at low population abundances in the N-limited community experiment (Fig. 5.1a) were neutral competitors for N. We have not investigated the traits of these diatom species, however, and hence we cannot ascertain their neutrality.

Conclusions

Our study illustrates that empirical demonstration of neutral coexistence is far from trivial. In particular, our experimental results support the theoretical prediction (Chesson, 2000; Leibold and McPeck, 2006; Adler et al., 2007) that species which appear to be neutral competitors in one niche dimension (with similar R^* for P) can display stable species coexistence, if their competitive traits are differentiated in subtle ways across other niche dimensions (differential utilization of the light spectrum). Thus, although the use of neutral theory to explain seemingly random species distributions in multispecies communities is tempting, we argue that in-depth analysis of species traits is required because unexpected niche-based forces can still be at play.

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