

## Review

## Harmful algal blooms: How strong is the evidence that nutrient ratios and forms influence their occurrence?

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## ABSTRACT

There is a perception that anthropogenically-driven changes in nutrient supply to coastal waters influences the abundance, frequency and toxicity of harmful algal blooms (HABs) through a change in the form or ratio of nutrient that limits phytoplankton growth. If nutrient concentrations are not limiting for growth, then ratios do not influence floristic composition. At non-limiting concentrations, evidence that alteration of nitrogen: phosphorus (N:P) ratios has stimulated HABs is limited, and primarily based on hypothesised relationships in relatively few locations (in particular: Tolo Harbour Hong Kong and Dutch Coastal Waters). In all cases, an unequivocal causal link between an increase in HABs (frequency, magnitude or duration) and change in N or P as the limiting nutrient is difficult to establish. The silicon (Si) limitation hypothesis is generally supported by experimental evidence and field data on the nuisance flagellate *Phaeocystis*. We found little evidence that high N:Si ratios preferentially promote harmful dinoflagellates over benign species. Laboratory studies demonstrate that nutrient ratios can influence toxin production, but genus and species specific differences and environmental control make extrapolation of these data to the field difficult. Studies of the role of dissolved and particulate organic nutrients in the growth of HAB species, while limited, demonstrate the potential for organic nutrients (especially organic N) to support the growth of a range of HAB species. There is a clear need for better understanding of the role of mixotrophy in the formation of HABs and for studies of HAB and non-HAB species in competition for environmentally realistic concentrations of organic nutrients.

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### 1. Introduction

A major factor governing the growth of different species of phytoplankton is the availability of mineral and/or organic nutrients. During the early part of the 20th century, an increase in the human population, industrialisation, and intensification of agriculture has increased the supply of nitrogen (N) and phosphorus (P) to coastal marine waters (Jickells, 1998). Fertilisers, atmospheric inputs, sewage, and agricultural wastes all contribute to this increase. These anthropogenic nutrients have the potential to stimulate phytoplankton production that in turn may have undesirable effects on coastal marine ecosystems and the human use of

them. This is the sequence of eutrophication (Nixon, 1995; Gowen et al., 2008).

A subset of species that make up the phytoplankton may be harmful to human health (e.g. through the production of natural biotoxins), or to human use of the ecosystem (e.g. causing mortality of farmed fish and restricting the harvesting of shellfish). The species that cause harm are widely referred to as “Harmful Algae” and the term “Harmful Algal Bloom” (HAB) is commonly used to describe their occurrence and effects. A link between anthropogenic nutrient supply and the appearance of harmful algae (HABs) has been proposed (Hallegraeff, 1993; Anderson et al., 2002; Heisler et al., 2008; Conley et al., 2009). The view that HABs occur in response to enhanced nutrient loading has recently been reviewed by Gowen et al. (2012) who found that the evidence relating HABs to anthropogenic nutrient enrichment was often equivocal.

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It is widely accepted that in coastal waters, it is the availability of dissolved inorganic N as ammonium ( $\text{NH}_4^+$ ), nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ) that is most likely to constrain (limit) phytoplankton growth (Ryther and Dunstan, 1971; Howarth and Marino, 2006). However, phosphorus (P) as ( $\text{PO}_4^{3-}$ ) is the limiting nutrient in some parts of the Baltic Sea (Andersson et al., 1996), the Eastern Mediterranean (Krom et al., 2004) and in the Pearl River estuary in southern China (Yin et al., 2001; Xu et al., 2008). A switch from spring P to summer N limitation has also been demonstrated in some locations (Conley, 1999). A meta-analysis of ecosystem response by Elser et al. (2007) suggests that the synergistic effects of combined N and P enrichment are more widespread in marine systems than previously thought. In addition to N and P, the concentration of silicate (Si) in coastal waters is important in governing diatom growth, since diatoms require Si for cell wall (frustule) formation. Hence, rather than being dependent on a simple elevation in coastal nutrient concentrations, the ratios of different nutrients and the form of these nutrients may influence HAB development. Nutrient ratios have also been suggested to influence the amount and rate of toxin generation by biotoxin producing species (Fehling et al., 2004; Granéli and Flynn, 2006).

The potential consequences of changing nutrient ratios for the growth of HAB species are based on the nutrient ratio hypotheses (Officer and Ryther, 1980; Tilman, 1977). These hypotheses purport that a perturbation in the nutrient supply ratio will result in the environmental selection of particular phytoplankters, potentially favouring harmful species (Smayda, 1990; Heisler et al., 2008). As human activity is thought to have increased N and P loads to coastal waters by different proportions (Falkowski, 2000; Conley et al., 2009), resultant changes in the N:P ratio provide a possible mechanism for an anthropogenically mediated increase in HABs.

Generally, Si is less influenced by anthropogenic activity than N and P. Hence in some regions of restricted exchange such as the southern North Sea (Smayda, 1990), the Baltic (Rahm et al., 1996), the Kattegat (Smayda, 1990) and Chesapeake Bay, USA (Conley and Malone, 1992), increases in the coastal inorganic N:Si ratio have been demonstrated. This potentially leads to a switch from diatom to dinoflagellate dominated communities (Officer and Ryther, 1980), the latter containing a greater proportion of HAB species.

The chemical form of nutrients may also be important. Phytoplankton generally exhibit preferential uptake of more reduced forms of N such as  $\text{NH}_4^+$  (Dortch, 1990; Flynn et al., 1993; Rees et al., 1995) that may be anthropogenically introduced into coastal waters (Eppley et al., 1979) as well as though *in situ* production within the food web (Davidson et al., 2005). The bioavailability to marine phytoplankton of natural and anthropogenic dissolved organic nutrients is also increasingly recognised, with a number of studies suggesting significant lability (Antia et al., 1991; Carlsson et al., 1993; Bronk, 2002; Lønborg et al., 2009a). The nitrogenous nutrient urea has been linked, through fertiliser runoff, with the development of coastal HABs (Glibert et al., 2006, 2008).

In summary, the hypothesis that HAB species abundance (and toxicity) is related to anthropogenically governed nutrient ratios in coastal waters, and/or the form (quality) of this nutrient, is frequently proposed in the scientific literature (Smayda, 1990; Paerl, 1997; Anderson et al., 2002; Glibert et al., 2005, 2006; Masó and Garcés, 2006; Heisler et al., 2008). However, while conceptually appealing, the nutrient ratio/HAB hypothesis is underpinned by a relatively limited number of studies. Moreover, the hypothesis is being challenged on theoretical grounds, with Flynn (2010) using a modelling to argue that phytoplankton growth on N and P is related to intracellular concentrations. This paper reviews the seminal papers to assess the breadth of evidence that underpin the hypothesis that anthropogenically influenced nutrient ratios and nutrient quality are key to HAB development and toxicity.

## 2. Nutrient – growth theories

Nutrients may 'limit' both the growth and the yield of phytoplankton populations. The former relates to the rate of increase of biomass, and the latter to the absolute amount of biomass generated per unit of nutrient available. The relationship between nutrients and populations of micro-organisms can be described by a number of theories. Application of these theories typically requires their articulation in mathematical form, for inclusion within modelling frameworks. Such models may then be used to predict the yield or rate of growth of a phytoplankton population given a certain nutrient supply.

The simplest "Monod" theory was developed for organic carbon-limited bacterial growth (Monod, 1942) and was subsequently applied to phytoplankton by Dugdale (1967). The rate of uptake of dissolved nutrient (per unit biomass) is described by a rectangular hyperbolic function of ambient extracellular concentration. Growth rate is directly proportional to uptake rate and increases with increasing ambient nutrient concentration up to a maximum. Both uptake and growth cease when the ambient limiting nutrient is exhausted. The Monod model does not account for cellular storage of nutrients. It is too simple to give a good description of laboratory growth of single species populations but may well be a reasonable approximation for assemblages in the sea (Davidson, 1996).

"Cell-quota" theory (Droop, 1968, 1983) is more sophisticated in that algal growth rates are controlled by cellular concentrations (cell quotas) of nutrients. The cellular quota,  $Q$ , of a nutrient (atomic nutrient element (atom organic carbon (C))<sup>-1</sup>), can vary between limits defined by the minimum or subsistence quota ( $k_Q$ ) and the maximum cell quota ( $Q_{\text{max}}$ ). The quota allows for cellular storage of a nutrient and so buffers against the effects of ambient change. In marine waters, the ratio of the maximum cell quota: subsistence quota ( $Q_{\text{max}}/k_Q$ ) is typically lower for N and Si (2–4) compared to P (5–90) and is part of the reason why marine phytoplankton tend to be N rather than P limited (Harrison et al., 1990). It also explains why a phytoplankton culture can continue to increase its biomass for many generations after P has been exhausted from the culture medium.

Cell quota based theory, or variants of it, has been used to describe a large range of steady state and transient growth dynamics (Davidson et al., 1993; Davidson and Cunningham, 1996; Tett and Droop, 1988). Most models assume a single limiting nutrient based on a threshold approach (Droop, 1974). The limiting nutrient being the one in least supply relative to cellular chemical composition and the cells' requirements for growth. The ratio  $k_{Q1}/k_{Q2}$  determines the relative limitation by nutrients 1 and 2. Thus, if the ratio of ambient nutrients 1 and 2 is  $<k_{Q1}/k_{Q2}$  then nutrient 1 may be limiting. However, while conceptually appealing such models are difficult to apply to natural systems because of the difficulties in measuring the cell quota in the field.

While at any one instant there is only a single yield limiting nutrient, the existence of dual nutrient limitation is consistent with the above theory when instantaneous N and P limitation are approximately equal in frequency and severity and hence when increases in phytoplankton biomass require N and P to be added together (Elser et al., 2009).

A further class of models employ complex mechanistic explanations of growth and incorporate more realistic accounts of the main biochemical processes and pools within cells (e.g. Davidson and Gurney, 1999; Flynn and Hipkin, 1999; Flynn, 2001, 2005). These parameter rich models often allowing representation of processes such as surge nutrient uptake (Conway et al., 1976; Mitra et al., 2003), but still retain the approach of the Monod and Quota models of using nutrient ratios to determine 'limitation'.

Finally, mixotrophy is increasingly regarded as an important process for phytoplankton nutrition (Raven et al., 2009). It presents a significant challenge to characterise theoretically and quantify experimentally. Mixotrophy is difficult to incorporate into models and simpler more tractable model formulations are unable to recreate the simulations of more complex models (Flynn and Mitra, 2009; Mitra and Flynn, 2010).

### 3. Measuring nutrient ratios

The need for evidence to test the HAB–nutrient ratio hypotheses presents a number of practical issues, such as how best to measure nutrient ratios and what nutrients should be used to derive the ratio. As mentioned above, ratios based on dissolved inorganic nutrients may only be relevant for obligate autotrophs. For mixotrophic species, ratios of total available nutrients may be more appropriate. Traditionally, the focus has been on dissolved inorganic N, P and Si, with N as nitrate before ammonium could be measured successfully. The ratios of maximum winter dissolved inorganic nutrients may help to assess which nutrient would potentially become limiting when nutrients are actively utilized during the spring bloom. The relevance of this approach for summer HAB species is questionable, and is restricted to high latitude waters with an obvious ‘winter’ period. Summer nutrient ratios may be more relevant to the development of HABs, but are difficult to measure accurately as limiting nutrients are rapidly recycled (Davidson et al., 2005). Competition by bacteria for nutrients has also been proposed as a mechanism that controls the form of nutrient limitation available to phytoplankton (Danger et al., 2007). Finally, the use of ratios of total N and total P (in the appropriate season and especially for oligotrophic aquatic systems) as measures of nutrients within algal cells is sometimes proposed, but such bulk measurements may be influenced by other suspended particulate matter.

Ratios of input fluxes from human discharges and land runoff have been used to assess the anthropogenic enrichment of coastal waters. However, these do not generally take into account the cycling of nutrients in estuaries (including: the equilibrium dynamics between dissolved available phosphate, bound phosphate and organic phosphorus; the dissolution of Si, and zooplankton grazing and microbial regeneration and rapid utilisation of  $\text{NH}_4^+$  denitrification) or the bioavailability of organic nitrogen compounds. Therefore, riverine loadings may not reflect the true ratio of nutrients. As ‘natural’ inputs (upwelling, diapycnal mixing, and benthic flux) are often hard to measure, few reliable data exist on the true nutrient ratios experienced by phytoplankton.

It is possible to determine nutrient uptake ratios from measurements of uptake using bulk chemistry or stable isotopes. Such measurements are valuable in assessing the uptake of different forms of N (Glibert and Capone, 1993) but are not routinely undertaken. While the term HAB implies a bloom, harmful phytoplankters (particularly those that produce biotoxins) do not necessarily have to ‘bloom’ or dominate the biomass to be harmful. This makes the field application of N uptake techniques problematic (Collos et al., 2007; Li et al., 2010). Moreover, determination of the rate of P and Si uptake is even more challenging, requiring the use of radioisotopes, and is conducted even less frequently.

### 4. Nitrogen to phosphorus ratio

Redfield (Redfield, 1958; Redfield, 1963) observed that chemical composition of plankton tends towards an atomic C:N:P ratio of 106:16:1. Redfield saw that since it was mainly the mineralization

of plankton-derived organic matter that resupplied the ambient pools of inorganic N and P, then the molar ratio of nitrate to phosphate should also tend to 16:1. The Redfield ratio of 16:1 for the molar ratio of ambient concentrations of dissolved inorganic N to P is widely used to infer which nutrient is likely limit the yield of a phytoplankton population. A ratio <16:1 is taken to indicate N limitation and a ratio >16:1 indicates P limitation. However, as acknowledged by Redfield, the ratio is a general basin wide and seasonal average.

Phytoplankton often appears to have ratios that approximate Redfield but can display a wide range of cellular composition. Geider and La Roche (2002) reported particulate N:P ratios in the range of 5–34. According to Klausmeier et al. (2004), changes in the cellular storage of nutrients is one reason for the large variation in nutrient stoichiometry in phytoplankton but this is additional to variability in stoichiometry brought about by changes in structural components (e.g. nucleic acids, proteins and pigments) for which the range in N:P is 7.1–43.3. Examples of large ranges in cellular composition include an observed C:N ratio of 28 for the eustigmatophyte *Nannochloropsis oculata* (Flynn et al., 1993) and C:N:P ratios ranging from 682:66:1 to 88:14:1 for the haptophyte *Pavlova lutheri* (Tett et al., 1985). This wide variation was due to different forms of nutrient limitation (N or P) in culture. Similar variability in ratios has been shown in euglenoids, dinoflagellates, chlorophytes, cryptomonads, diatoms, pelagophytes, and cyanobacteria (Tett et al., 2003).

The use of the Redfield ratio to differentiate between N and P limiting conditions has been questioned. Geider and La Roche (2002) suggested that the critical N:P ratio which marks the transition from N to P limitation is between 20 and 50 mol N:mol P but based on a typical biochemical composition (i.e. the critical N:P ratio for nutrient replete phytoplankters) is between 15 and 30. Since different species have different cellular requirements, some species may be P limited while others are N limited. Zang and Hu (2011) demonstrated different optimal N:P ratios for a number of phytoplankters depending on the form of the N source. A simple relationship between floristic composition and N:P ratio is therefore insufficient to demonstrate a causal link between them.

In light of the caveats above, we now evaluate the evidence linking anthropogenically generated shifts in nutrient ratios to HABs in a number of geographical locations where this link has been proposed.

#### 4.1. N:P ratios and summer *Phaeocystis* in the southern North Sea

The prymnesiophyte *Phaeocystis* is a nuisance species in coastal waters (Lancelot et al., 1987; Schoemann et al., 2005). Using laboratory cultures, Riegman et al. (1992) demonstrated that *Phaeocystis* sp. was a poor competitor for P, being out competed in multi-species experiments by *Emiliania huxleyi* and *Chaetoceros socialis*. In contrast, it was a good competitor in N limited conditions. This implies that a low N:P ratio would favour *Phaeocystis* sp. Riegman (1995) used these results to argue that the shift from P to N limiting conditions in Dutch coastal waters (total N: total P ratios decreased from 38 to 13 during the late 1970s and 1980s in the Marsdiep region of the Dutch Wadden Sea) was the reason that “novel summer blooms of *Phaeocystis* appeared in the late seventies”. This assertion, with over 150 citations, has become one of the major studies underpinning the nutrient ratio – HAB hypothesis.

There are four points which need to be considered in relation to the *Phaeocystis*-N:P ratio hypothesis. Firstly, summer *Phaeocystis* blooms are not a recent phenomenon in the region (Cadée and Hegeman, 1986; Peperzak, 1993). In his papers, Riegman stated that a perturbation in the N:P ratio led to an increase in the magnitude and duration of summer blooms (Riegman et al., 1992;

Riegman, 1995). However, Riegman (1995) also notes “the sudden appearance of *Phaeocystis* summer blooms” and “Novel summer blooms of *Phaeocystis* appeared in the late seventies”. This has led some subsequent works (for example the extensively quoted review of Hallegraeff (1993), to state that “this species first appeared in Dutch coastal waters in 1978” and Smayda (1990) to state that “mass occurrences began in 1977...” incorrectly implying that the change in N:P ratio generated summer *Phaeocystis* blooms in Dutch coastal waters.

The second point is that there are contrasting interpretations of available data. Riegman et al. (1992) interpreted the data in Fig. 1 as showing that “*Phaeocystis* became more dominant at average summer N:P ratio below 16”. However, in our opinion the data are more equivocal, as *Phaeocystis* was only elevated in 5 out of the 8 years post 1977 when N:P was in this range. The relationship between nutrients and the phytoplankton community composition in the Dutch Wadden Sea was also discussed by Philippart et al. (2007) who also found the data to be less clear cut than Riegman’s interpretation. Philippart et al. (2007) found some significant relationships between the concentrations and ratios of inorganic N, P and Si to community structure but that these relationships were often quite weak. Furthermore, in contrast to Riegman et al. (1992), Weisse et al. (1986) concluded (on the basis of field observations in the German Wadden Sea) that *Phaeocystis* sp. had lower inorganic nutrient demands, especially for P compared to diatoms, and could grow when the P concentration was 0.2  $\mu\text{M}$ . Veldhuis and Admiraal (1987) and Veldhuis et al. (1991) also found that *Phaeocystis* colony formation occurred at P concentrations  $<0.2 \mu\text{M}$ . Finally, mesocosm results (Brussaard et al., 2005) in coastal North Sea waters did not show a marked difference in the percentage contribution that *Phaeocystis* spp. made to the phytoplanktonic community under N or P limited conditions.

The third point is that the magnitude of the change in the N:P ratio in the region is unclear. Riegman (1995) indicated that total molar N and P was used to assess changes in the N:P ratio. He argued that it was not possible to identify the nature of the controlling factor on the basis of dissolved inorganic nutrient concentrations and ratios, but that ratios of total molar N:P might give some indication of the potential controlling factor when light is not limiting. The reason why the former cannot be used but the latter can is not made clear by Riegman (1995) and as discussed above, there are interpretational difficulties with both. However, assuming limitation of a particular inorganic nutrient based on ratios of ambient concentrations presupposes that nutrients will be taken up in the Redfield ratio, and that the Redfield ratio denotes

the transition from N to P limitation. As discussed above, neither is necessarily the case.

The fourth point to consider in relation to the *Phaeocystis*-N:P ratio hypothesis is the extent to which nutrients control summer growth in Dutch coastal waters. For the inner regions of the Wadden Sea, Postma and Rommets (1970) found nutrients to be rarely a limiting factor for phytoplankton growth in either spring or summer. Both Cadeé and Hegeman (1991) and Riegman et al. (1992) related spring *Phaeocystis* blooms in the region prior to the change in the N:P ratio to light limitation. Indeed, Colijn and Cadée (2003) were of the opinion that in the Wadden Sea and the Marsdiep region in particular, irradiance was more limiting for the production of phytoplankton biomass and there was only slight nutrient limitation between May and July.

In summary, the evidence does not preclude the role of nutrient ratios in partly controlling the phytoplankton community in Dutch coastal waters but other factors may be important or dominant, and the strength of the link between *Phaeocystis* blooms and N:P ratios is not convincing. This view is consistent with the original work of Riegman et al. (1992) who indicated a more detailed analysis of field data from multiple geographic regions was required to confirm their laboratory results. This suggestion remains true today. Contemporary studies (e.g. Lancelot et al., 2009) still suggest that the drivers of long term fluctuations in *Phaeocystis* in the region are unclear, with the dynamics of *Phaeocystis* blooms in continental coastal waters of the southern North Sea also having been related to climate (Breton et al., 2006; Gieskes et al., 2007).

#### 4.2. N:P ratios and red tides in Hong Kong waters

The term “red tide” has gained wide usage for the discolouration of the sea (any colour) by high biomass blooms of any species. The six most common red tide species in coastal waters of Hong Kong, China in descending order were: *Noctiluca scintillans*, *Gonyaulax polygramma*, *Skeletonema costatum*, *Myrionecta rubra*, *Prorocentrum minimum*, and *Ceratium furca*. Of these, the heterotrophic dinoflagellate *N. scintillans* accounted for 40% of the red tide events and the ciliate *M. rubra* forms autumnal red tides (Yin, 2003). Both of these organisms are grazers, are not harmful, and are not directly influenced by nutrient ratios.

Tolo Harbour (Hong Kong) is a small enclosed water body within which a large number of red tides have occurred. Hodgkiss and Ho (1997) presented a data set showing that between 1982 and 1989, the N:P ratio in Tolo Harbour decreased from  $\sim 20$  to 11. This was accompanied by a shift from diatoms to dinoflagellates and a statistically significant increase in the annual occurrence of red tides from  $\sim 10$  to 20. They suggested that the change in the ambient N:P ratio was a possible reason for this correlation. Hodgkiss and Ho (1997) based their conclusion on earlier laboratory experiments (Ho and Hodgkiss, 1993) that showed the cellular yield of eleven red tide organisms (10 dinoflagellates and 1 diatom) was optimized at low N:P molar ratios of between 4 and 16, indicating that these mainly dinoflagellate HABs have a high P requirement relative to N. However, it is not clear how they determined that the optimum N:P ratio for growth of *Noctiluca scintillans* since it is a heterotroph and does not take up inorganic N and P. Hodgkiss and Ho (1997) proposed that: “both long term and relatively short term changes in the N:P ratio are accompanied by increased blooms of non-siliceous phytoplankton groups” and further argued: “that although in classical Liebigian terms minimum amounts [of nutrients] can be limiting, nutrient ratios (such as N:P and Si:P) are far more important regulators”.

The putative response of HABs in Tolo Harbour to changes in N:P ratio reported by Hodgkiss and Ho (1997) has become one of the main pillars underpinning the N:P-HAB hypothesis and has

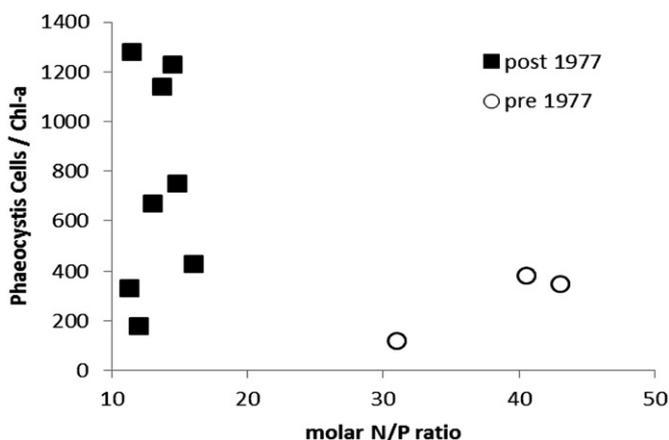
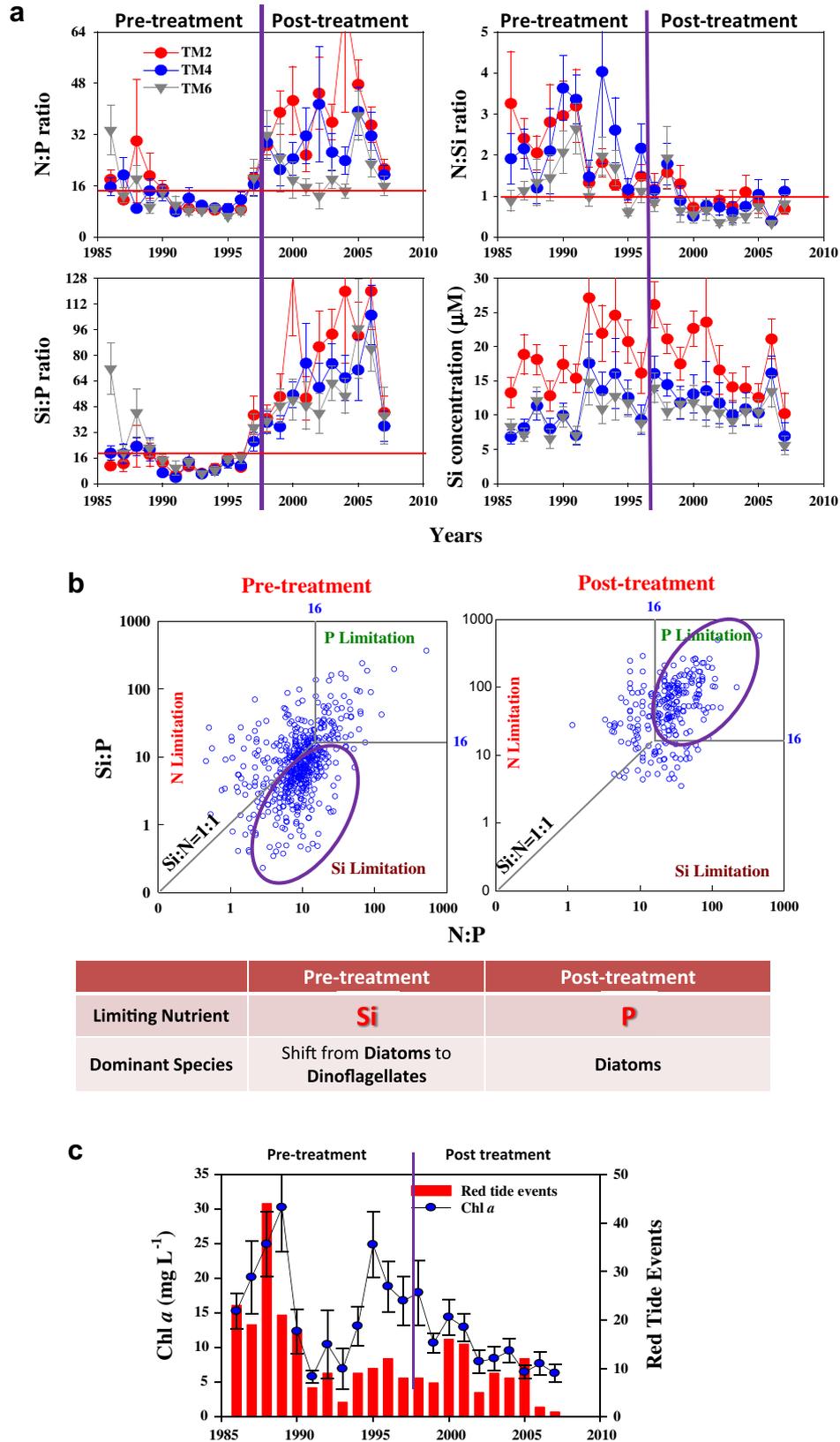


Fig. 1. The relationship between *Phaeocystis* and N:P ratio in the Marsdiep are of Dutch coastal waters. Redrawn from Riegman et al. (1992).



**Fig. 2.** a) Time series (1986–1998 = pre-diversion and 1998–2007 = post-diversion as denoted by the vertical line) of nutrient ratios and Si concentrations for 3 stations in inner Tolo Harbour, TM2, TM4, TM6. Horizontal lines indicate Redfield ratios of N:P = 16:1, Si:P = 16:1 and N:Si = 1:1. Redrawn from Harrison et al. (2012). b) Si:P versus N:P molar ratios for pre- (1986–1997) and post (1998–2007) sewage diversion in Tolo Harbour. The number of data points in each quadrant indicate the tendency for potential limitation by a particular nutrient. Redrawn from Harrison et al. (2012). c) Pre- and post-treatment chlorophyll concentrations and red tide events in inner Tolo Harbour. Vertical line indicates the time of sewage diversion in 1998. Redrawn from Harrison et al. (2012).

frequently been cited as evidence supporting the hypothesis (e.g. Anderson et al., 2002; Masó and Garcés, 2006; Cartersen et al., 2007). However, a large scale sewage diversion in Tolo Harbour in 1998 provided an experiment to test the nutrient ratio-HAB hypothesis. Analysis of a 25 year time series spanning the sewage diversion shows that red tide occurrences did not decrease when there was a significant increase in N:P ratios (Harrison et al., 2012).

Since sewage has a low N:P ratio of  $\sim 10$  (i.e. is P-rich), the ambient N:P ratio in inner Tolo Harbour (Stations TM2, TM4, TM6) decreased from  $\sim 16$  to 10 from 1986 to 1997. Following sewage diversion in 1998, the N:P ratio increased above Redfield (N:P  $\geq 30$ ) immediately but had fallen to approximately Redfield in 2007 (Fig. 2a). After 1998, inorganic N loading decreased by 40% and inorganic P loading by 75% and the Si:P and Si:N ratios had increased  $\sim 10$  and 5-fold, respectively (Xu et al., 2010). Before the sewage diversion there was a tendency for potential Si limitation and possibly N limitation as denoted by the horizontal lines of Redfield ratios of 16Si:1P and 1N:1Si in Fig. 2a, but after this the tendency for potential P limitation increased because of the large reduction in P (Fig. 2a,b).

Hodgkiss and Ho (1997) argued that a low N:P ratio favoured HAB species but from 1991 the annual occurrence of red-tides fell to  $\leq 10$  (Fig. 2c) at a time when the N:P ratio was  $\leq$  Redfield. Furthermore, red-tide frequency remained low ( $\leq 12 \text{ yr}^{-1}$ ) following the 1998 reduction in discharge. Therefore, the extended 25 year time series provides no evidence of a N:P ratio driven change in the occurrence of HABs. In addition, red tide events were not coupled with phytoplankton biomass (as chlorophyll, Chl-*a*) since red tides decreased seven years before the sewage diversion, while Chl-*a* decreased after the diversion as expected (Fig. 2c).

Wong et al. (2009) suggested that the year (1988) with the highest number of red tides recorded may have been due to this being a low wind year with 6 weak typhoons, while the year (1993) with the fewest red tides was a high wind year with 9 large typhoons. For Victoria harbour in Hong Kong, the highest number of red tides also occurred in 1988. These two harbours differ hydrodynamically. Tolo Harbour is always stratified and is slowly flushed, while Victoria Harbour is generally vertically mixed and rapidly flushed (Xu et al., 2010). This suggests that larger scale physical forcing, rather than local factors may have been the cause of the high number of red tides in 1988. In summary hydrodynamic conditions, Si limitation, grazing and large scale physical forcing are likely to play a more important role in determining the occurrence of red tide events than N:P ratios in Tolo Harbour and other Hong Kong waters.

#### 4.3. Other HAB events linked to N:P ratios

The role of N:P ratios in governing freshwater cyanobacteria has been long debated. Smith (1983) and Smith and Bennet (1999) argued that low N:P ratios were responsible for cyanobacteria dominance. However, Reynolds (1999) and Downing et al. (2001) questioned this, arguing that relationships were coincidental rather than causal, and that P was a better predictor of cyanobacterial dominance. In marine waters there are fewer locations where anthropogenic modification of nutrients may have influenced harmful cyanobacterial blooms. Most debate has focussed on the Baltic Sea, where diazotrophic cyanobacteria, through their ability to fix  $\text{N}_2$  gas, are able to utilise the available P when the growth of other organisms is N limited. *Nodularia spumigena*, *Anabaena* spp., and *Aphanizomenon flos-aqua* are prominent HAB species (although the latter is thought to be non toxic in that region).

The occurrence of cyanobacterial blooms in the Baltic is not a new phenomenon. Bianchi et al. (2000) used fossil records to

demonstrate their occurrence during the last 7000 years. An increase in bloom frequency and biomass has been reported in recent decades (Bianchi et al., 2000; Vahtera et al., 2007; Pliński et al., 2007), and has coincided with 5–10 and 10–20 fold increases in anthropogenic N and P respectively since the beginning of the 20th century (Larsson et al., 1985). This has led some to suggest that anthropogenic nutrients have elevated the N:P ratio and increased the extent and intensity of blooms (Pliński et al., 2007). While some spatial and seasonal variability of nutrient ratios and nutrient limitation exist (Granéli et al., 1990; Tamminen and Andersen, 2007), the bulk of this sea is thought to be N limiting (low N:P ratios) for phytoplankton growth. When combined with low salinity and suitable weather conditions (Kanoshina et al., 2003), this generates conditions that promote cyanobacterial blooms.

Most anthropogenic nutrients arrive at the coast and while some blooms occur in coastal waters, major blooms occur offshore (Vahtera et al., 2007). Many authors now argue that offshore blooms are regulated by internal nutrient cycling processes. As it declines, the N limited spring bloom sinks to bottom waters where it contributes to hypoxia that promotes sediment P release. This P reaches surface waters through annual turnover or summertime upwelling and supports summer diazotroph blooms. The causes of cyanobacterial blooms in the Baltic Sea are therefore more complicated than just a matter of low N:P ratio (Tamminen and Andersen, 2007). Elevated N could support increased spring (non cyanobacterial) bloom production and hence promoting subsequent cyanobacterial blooms through enhanced P release from sediments. Therefore, there is little evidence that changes in the N:P ratio resulting from anthropogenic nutrient enrichment has directly influenced the main cyanobacterial blooms in the Baltic. Both meta-analysis and modelling (Vahtera et al., 2007; Wulff et al., 2007) suggest that it is the N and P load rather than their ratio which is important in influencing the occurrence of Baltic HABs.

Our final example comes from the study of toxic dinoflagellates in Tunisian lagoons (Romdhane et al., 1998) that was cited as an example of nutrient ratios influencing HABs by Anderson et al. (2002). Blooms of *Alexandrium* sp. (a producer of paralytic shellfish toxins, Oshima et al., 1989) and *Karenia mikimotoi* (a fish killing species, Davidson et al., 2009) occurred during this study. Romdhane et al. (1998) undertook a statistical analysis of the relationship between the blooms and a range of potential environmental drivers. No significant relationships were found but importantly, nutrient ratios were not included in the analysis. The suggested relationship between the HABs and nutrient ratios was therefore qualitatively; based only on a suggestion that blooms develop seasonally in a location where N:P ratio was high (i.e. the opposite to Hodgkiss and Ho, 1997). While the authors linked this to anthropogenic influences, no direct evidence was provided in support of this link. Once again, the relationship between anthropogenic modification of N:P ratios and HABs is somewhat speculative and further quantitative studies would be required before more robust conclusions could be drawn for this location.

## 5. Silicate limitation of diatom growth

Officer and Ryther (1980) and subsequent studies have argued that the Si:N ratio determines the dominant group of phytoplankton. Si limitation in coastal waters is exacerbated by low concentration of Si relative to N and P in sewage effluent and fertilisers. Thus, in situations in which Si becomes limiting for diatom growth, diatoms are replaced by dinoflagellates or microflagellates (and most HAB species are flagellates). In areas receiving sewage effluent and/or fertilizer runoff, attention should therefore be focussed on Si:N and Si:P ratios.

Historically, Si concentrations in river water were well in excess of dissolved inorganic N and P concentrations but many rivers are now showing signs of a stoichiometric nutrient balance of Si:N:P = 16:16:1 or less due to the increases in N and P (Justic et al., 1995). The damming of river systems can lead to a net decrease in the amount of silicate reaching the coast and a decrease in the Si:N:P ratio potentially generating an increase in HABs (Admiraal et al., 1990; Conley, 1997).

While all diatoms require silicate for cell wall formation, species differ in their wall thickness and hence silica content varies. A mean N:Si not significantly different from 1 was proposed by Brzezinski (1985) but the point at which any nutrient mediated switch in community composition occurs is variable. Harrison and Davis (1979) conducted outdoor continuous cultures with natural assemblages receiving N:Si ratios of 3:1 (Si-limited) and 0.2:1 (N-limited). In the Si-limited conditions, there was a shift from the typical chain-forming centric diatoms (*Skeletonema*, *Chaetoceros*, *Thalassiosira*) to pennate forms (e.g. *Cylindrotheca*) and some very small *Chaetoceros* sp. Since the final assemblage was >50% diatoms, this indicates that many diatoms have low Si requirements and can maintain their population under moderate Si limitation. In some cases the ratio  $N_{kQ}:Si_{kQ}$  is  $\approx 2$ , (Tett et al., 2003) meaning that the cell would require twice as much N as Si. In agreement with this, Gilpin et al. (2004) found that flagellates began to dominate over diatoms in mesocosms in Trondheim fjord, Norway once the N:Si supply ratio exceeded 2 (although there were no significant changes in the relative abundance of HAB species present (Roberts et al., 2003)).

### 5.1. N:Si ratios and Spring *Phaeocystis* blooms in the Southern North Sea

Silicate limitation is thought to have resulted in an increase in the size and duration of spring *Phaeocystis* blooms (Cadée and Hegeman, 1986; Lancelot et al., 1987; Lancelot, 1990). Increased anthropogenic inputs of nutrients have resulted in excess N being available to flagellates and dinoflagellates after the Si dependent spring diatom bloom.

Mesocosm experiments have been used to explore the Si-limitation hypothesis. Egge and Aksnes (1992) working in the Bergen (Norway) mesocosm facility, found diatoms dominated when the silicate concentration was  $>2 \mu\text{M}$ . *Phaeocystis* spp. appeared after the bloom of other species, but not when Si concentrations were high. The specific reasons why *Phaeocystis* rather than other flagellates bloom when diatoms are Si limited is not fully understood, but may be related to an affinity for different forms of N and grazing resistance (Bradley et al., 2010; Lundgren and Granéli, 2010).

The above studies provide general support that *Phaeocystis* may grow and bloom when there is an excess of N and diatom growth is Si limited, but there are some counter examples. Escaravage et al. (1995) found that, in mesocosms in which the light and nutrient regimes resembled early summer conditions in Dutch coastal waters, *Phaeocystis* spp. out-competed diatoms in nutrient replete conditions. In culture experiments, Peperzak (1993) examined the influence of daily irradiance on growth rate and colony formation and concluded that below an irradiance threshold of  $100 \text{ W h m}^{-2} \text{ d}^{-1}$  cells of *Phaeocystis* spp. were small and there was no colony formation. Above this threshold, increased cell size and no colonies formed. Similarly, Peperzak et al. (1998) examined data from Dutch coastal waters in 1992 and concluded that the timing of *Phaeocystis* spp. blooms correlated with a daily light threshold and was not linked to Si limited diatom growth.

### 5.2. N:Si ratio in the German Bight

Anthropogenic enrichment has perturbed winter N:Si ratios in the German Bight. Radach et al. (1990) reported an increase in the

molar N:Si ratio from 1 to 2 in the late 1960s, to 4–8 in the early 1980s. However, the Si limitation hypothesis is not consistent with all observations in the southern North Sea. During 1988–1989, *Phaeocystis* spp. made up a larger proportion of the phytoplankton in East Anglian (UK), waters than in the German Bight, although N:Si ratios were higher in the latter (Tett et al., 1993; Tett and Walne, 1995).

The difficulty in relating changes in the phytoplankton community to Si limitation is evident from other studies in the German Bight. Analysis of the Helgoland time series led Hickel (1998) to conclude that there was evidence of nutrient enrichment, but the expected long-term trends in phytoplankton were not always evident. Recurrent 3–5 year cycles of diatom and flagellate biomass were apparent, but the three-fold increase in total phytoplankton was largely due to an increase in the winter biomass of nanoplankton. Since light limited phytoplankton growth during the winter, Hickel (1998) was of the opinion that: the flagellates were mostly heterotrophic and mixotrophic species  $<5 \mu\text{m}$  in size; their increase was not correlated with inorganic N, and the explanation for the increase in flagellates was not known but coincided with other large scale effects. Hickel (1998) concluded that once the nanoplankton component was separated from the autotrophic microplankton, and neither diatom nor dinoflagellate biomass showed a clear long term upward trend.

### 5.3. Changes in N:Si ratio and the toxic diatom *Pseudo-nitzschia* in the Gulf of Mexico

In the Mississippi River plume of the northern Gulf of Mexico, enrichment with N and a reduction in Si load have resulted in elevated N:Si ratios (Turner et al., 1998). Contrary to the expected perturbation of the floristic composition in favour of dinoflagellates, there has been an increase in diatoms, especially the genus *Pseudo-nitzschia* (Parsons et al., 2002), species of which produce domoic acid that causes amnesic shellfish poisoning (Fehling et al., 2004). These findings are consistent with other field data that suggest *Pseudo-nitzschia* and other lightly silicified diatoms (e.g. *Leptocylindrus* spp.) prosper in low Si condition (Fehling et al., 2006). Pan et al. (1996) studied the ratios of maximum to minimum cellular Si ( $Q_{\text{max}}$  to  $k_q$ ) for a range of diatom species in the laboratory. For *Pseudo-nitzschia multiseries*, the ratio was 15.3 compared to 1.1 for *Cerataulina pelagica* and 8.8 for *Coscinodiscus granii*. The authors related the high value (15.3) to luxury consumption and suggested that *P. multiseries*, (and perhaps species of *Pseudo-nitzschia* in general), were able to grow under a wide range of Si concentrations and was one explanation for its ubiquitous distribution.

### 5.4. Summary of the Si limitation hypothesis

While most studies are supportive of the Si limitation hypothesis in terms of promoting flagellate growth over that of diatoms, relatively few provide definitive evidence that HAB species, other than *Phaeocystis* (and even then with counter examples), prosper over benign species under these conditions. The suggestion that Si limitation of diatoms will promote HABs is therefore based on the greater proportion of dinoflagellates that are harmful compared to diatoms, rather than a weight of experimental evidence.

## 6. Nutrient composition and quality

The composition of the available N pool has been suggested to influence the development of HABs. Phytoplankton require a source of N which can be dissolved inorganic N, or organic N in the form of amino acids, urea or other bioavailable forms. Many studies have investigated the use of different forms of N by HAB species. In general, these have confirmed the preference for reduced N (Bates, 1976; Dortch, 1990), particularly by dinoflagellates that typically

proliferate in summer when regenerated, reduced forms of N are a large proportion of the available N pool. Examples include *Alexandrium* (Maguer et al., 2007), and *Prorocentrum minimum* (Lomas and Glibert, 1999; Fan et al., 2003).

Nitrate ( $\text{NO}_3^-$ ) is often regarded as the nutrient of choice for diatoms because it fuels the spring bloom in temperate waters, but  $\text{NH}_4^+$  may be important in promoting harmful diatom blooms. Suksomjit et al. (2009) and Tada et al. (2010) found that *Skeletonema*, that forms red tides in some parts of the world, grows up to 25% faster on  $\text{NH}_4^+$  compared to  $\text{NO}_3^-$ .  $\text{NH}_4^+$  may fuel the initial stages of *Skeletonema* bloom development and lead to a larger 'seed' population that is better able to utilize  $\text{NO}_3^-$  when  $\text{NH}_4^+$  is exhausted.

A number of studies have investigated the relative importance of  $\text{NO}_3^-$  versus  $\text{NH}_4^+$  as an N source for *Pseudo-nitzschia*. Bates et al. (1993) reported lowered growth rates of *Pseudo-nitzschia pungens* f. *multiseriata* at  $\text{NH}_4^+$  concentrations  $>220 \mu\text{M}$ , but such un-naturally high concentrations are likely to prove toxic to the cells (Azov and Golman, 1982). Similarly, Hillebrand and Sommer (1996) demonstrated  $\text{NH}_4^+$  inhibition for this species at  $160 \mu\text{M}$ . Studies conducted with concentrations typical of coastal waters generally find little influence of the form of the inorganic N source. Fehling (2004) investigated the growth of *Pseudo-nitzschia seriata* in laboratory culture when growing on (as limiting nutrient)  $\text{NO}_3^-$ ,  $\text{NH}_4^+$  or a 50:50 mixture of the two, and found no significant difference in the exponential phase growth rates or the cell yield (Fig. 3). Similarly, Thessen et al. (2009) found no clear pattern in how  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and urea (discussed more fully below) influenced growth rates of different *Pseudo-nitzschia* species. In contrast, Cochlan et al. (2008) found *Pseudo-nitzschia australis* exhibited a preference for  $\text{NO}_3^-$  over  $\text{NH}_4^+$ .

### 6.1. Dissolved organic and particulate nutrients

There are significant pools of dissolved organic N and P (DON and DOP) in coastal waters (Antia et al., 1991; Bronk, 2002; Karl and Björkman, 2002; Lønborg et al., 2009a). During summer in temperate waters (when concentrations of inorganic nutrients are low) DON and DOP may be the largest pools of fixed N and P and potentially important for phytoplankton nutrition. While it is generally accepted that phytoplankton can utilise a range of organic compounds, their quantitative importance in phytoplankton nutrition is uncertain (Antia et al., 1991; Caron et al., 2000) as much of the dissolved organic matter (DOM) pool may be refractory.

DOM can reach coastal waters by a number of pathways. Biotic contributions to the DOM pool include the release from

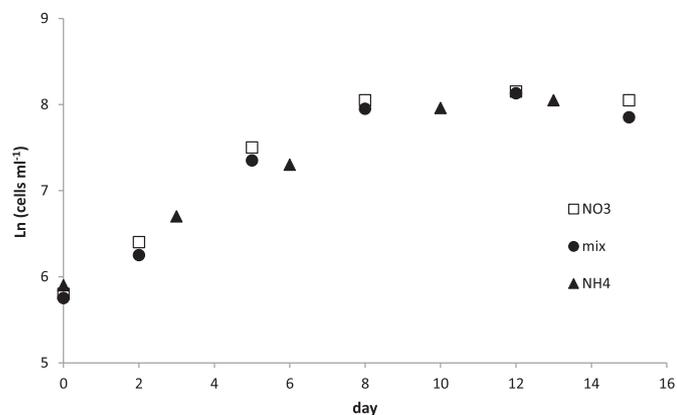


Fig. 3. Natural logarithm of *Pseudo-nitzschia seriata* cell density versus time in laboratory culture grown on  $\text{NO}_3^-$ ,  $\text{NH}_4^+$  and a 50:50 mix of the two nutrients.

phytoplankton (Pete et al., 2010), bacteria (Berman et al., 1999), heterotrophic protists (Lønborg et al., 2009b), mesozooplankton (Carlson, 2002) and viral action (Fuhrman, 1999). Abiotic inputs come from the atmosphere (Cornell et al., 1995), rainwater (Seitzinger and Saunders, 1999), or riverine delivery (Glibert et al., 2005). Utilisation of those compounds that are labile, perhaps mediated by prior bacterial remineralisation (Davidson et al., 2007) may make organic nutrients an important resource for phytoplankton.

A number of studies have focussed on utilisation of DOM by *Alexandrium* species. Stolte et al. (2002a) demonstrated DON uptake by *Alexandrium tamarense* and Gagnon et al. (2005) found that humic substances enhanced its growth rate. Loureiro et al. (2009c) found that adding natural marine DON to cultures containing nitrate enhanced the growth rate of *Alexandrium catenella*, and Fagerberg et al. (2009) drew similar conclusions for *Alexandrium minutum*, but also noted that (refractory) low molecular weight (LMW) DOM enhanced growth more than high molecular weight (HMW) DOM. However, HMW DOM (that is often assumed non-refractory) has been shown to support the growth of *Pseudo-nitzschia delicatissima* in culture (Loureiro et al., 2009a) and in field bioassays (Loureiro et al., 2009b).

DOM is utilised by a range of other HAB species. *Prorocentrum minimum* exhibited enhanced growth rate and cell yield following organic additions to cultures and mesocosms (Heil et al., 2005). DON has also been proposed as an N source for the brown tide species *Aureococcus anophagefferens* with several studies demonstrating preferential use of DON over inorganic N (e.g. Berg et al., 2002, 2003). Field data are consistent with this laboratory evidence; La Roche et al. (1997) observed large decreases in DON in parallel with bloom development in coastal waters of Long Island, USA. Glibert et al. (2007) suggested that bloom magnitude was related to prior DON availability in coastal bays of Maryland, USA. Dissolved organic phosphorus (DOP) has also been related to the occurrence of HABs, with high alkaline phosphatase activity during some HAB events (Glibert and Burkholder, 2011). Direct use of DOP has also been investigated in culture experiments and Oh et al. (2002) found that both *Alexandrium tamarense* and *Gymnodinium catenatum* from Hiroshima Bay, Japan utilized DOP.

Finally, the ratio of DOC to DON has also been linked to some HABs. For example, elevated DOC:DON ratios ( $>10$ ) occurred during blooms of *Aureococcus anophagefferens* in Chesapeake Bay (Lomas et al., 2001). However, while elevated DOC environments may favour *Aureococcus*, it is less clear that the DOC:DON ratio is relevant, as the high DOC concentrations were unlikely to be fully consumed.

The link between DOM and HAB species growth it is not universal. For example, Nishimura (1982) undertook culture experiments using *Gymnodinium* type-65 and *Chattonella antiqua* supplemented with organic rich water from the vicinity of fish farms. He found that *Gymnodinium* grew well, but *C. antiqua* did not grow with organic additions. In addition, reviews of the influence of fish farming on HAB occurrence in Scottish waters (Tett and Edwards, 2003; Rydberg et al., 2003; Smayda, 2005) concluded that there was no evidence of aquaculture wastes (including both inorganic and organic nutrients) stimulating HABs. A similar conclusion was reached for fish farms in Tolo Harbour by Yin et al. (2007).

The nitrogenous compound urea [ $\text{CO}(\text{NH}_2)_2$ ] is a potentially important DON source. Glibert et al. (2006) reported that urea use had increased in recent years and worldwide represented  $>50\%$  of total global nitrogenous fertiliser usage, although usage varies regionally. Unhydrolyzed urea can be lost to surface runoff and urea concentrations in receiving estuaries and coastal waters can potentially be enhanced by these land-based inputs. Urea

concentrations in seawater are typically less than those of  $\text{NO}_3^-$  or  $\text{NH}_4^+$  (Bronk, 2002) but local transient increases occur, particularly when rainfall runoff occurs from heavily urea fertilised areas such as the watersheds of Chesapeake Bay, Santa Cruz California USA, and the Knysna Estuary, South Africa (Solomon et al., 2010). Glibert et al. (2006) suggested that in some regions of the world where urea dominated agricultural applications of N, there was a correlation with increasing numbers of HABs. However, this link is primarily based on a comparison of global maps of urea usage in the 1960s and in 1999 and the occurrence of dinoflagellate species causing PSP or documented cases of PSP at similar times. Such maps are difficult to interrogate and suggestions of an increase in PSP species/incidents may also be related to a number of other factors including: under representation of historical PSP events; increased recent monitoring efforts; climate change; species introductions.

The work of Glibert et al. (2006) has generated considerable debate on the role of urea in stimulating HABs. Solomon et al. (2010) summarised many of the laboratory studies of urea utilisation by HAB species, finding that urea supported both higher and lower growth rates compared to rates with  $\text{NO}_3^-$  or  $\text{NH}_4^+$  as the substrate. To date, few *in situ* or mesocosm experiments have been conducted to evaluate the role of urea, although Kana et al. (2004) demonstrated that organic N (including urea) was more important than inorganic N in stimulating growth of the *Aureococcus anophagefferens*. The ability of urea to support harmful blooms was also demonstrated in mesocosms by Sanderson et al. (2008) who observed that urea accounted for the largest percentage of total N uptake compared to other N substrates (up to 65 times greater) during a bloom of the nuisance flagellate *Phaeocystis*.

Direct field observations of urea promoting HABs are also relatively rare, but this has been demonstrated in some locations. Lomas et al. (2001) showed that  $^{15}\text{N}$  urea supported the development of *A. anophagefferens* blooms in coastal bays of Maryland and Long Island. Similarly, Li et al. (2010) demonstrated significant urea usage by a bloom of *Karenia mikimotoi* in Chinese waters. Collos et al. (2007) observed that urea was capable of supporting up to 59% of *Alexandrium catenella* growth in the Thau Lagoon, France and that, concentrations above a threshold level (0.5–1.5  $\mu\text{M}$ ) could potentially trigger blooms. This is consistent with the results of Glibert and Terlizzi (1999) who also suggested a 1.5  $\mu\text{M}$  urea threshold for the development of dinoflagellate blooms in Chesapeake Bay. However, Collos et al. (2007) stated that comparison of his results with those of Glibert and Terlizzi (1999) should be conducted with caution due to complicating factors such as the balance of production, consumption and the relationship between urea and other components of the dissolved N pool.

Few studies have directly considered the role of particulate organic matter (POM) as a substrate for microalgal growth. Nevertheless, given that much of the recycled nutrient pool is derived from remineralised algal biomass, the POM pool must represent a source of utilisable organic matter. Isotope based studies (Fichez et al., 1993) indicate that terrestrial POM persists in coastal waters, suggesting that it is relatively inaccessible to phytoplankton. In contrast, Montagnes et al. (2008) argue that POM may be important for mixotrophic and heterotrophic dinoflagellates.

In summary, the number and breadth of studies on the importance of DOM as a nutrient source for phytoplankton are small compared to studies of inorganic nutrients. However, evidence from cultures and the few field studies that have been conducted suggests that DOM may be important for some HAB species.

## 7. Nutrient limitation and toxin production

The harm produced by many HAB species is due to their production of natural biotoxins that impact humans and other mammals. Intoxication often follows consumption of shellfish that have filter fed on a HAB species and bio-accumulated toxin. Toxin production is not necessarily continuous or of a constant amount per cell. This makes understanding the factors governing toxicity important. The role of nutrient ratios in influencing toxin production is discussed below, principally for the two genera, *Pseudo-nitzschia* and *Alexandrium* that have received the most detailed investigation.

Bates et al. (1993) observed that *Pseudo-nitzschia pungens* required a high external supply of inorganic  $\text{NO}_3^-$  to produce the toxin domoic acid (DA). This is consistent with DA being an amino acid, hence requiring N for its synthesis. Although a few exceptions exist (Garrison et al., 1992), subsequent laboratory studies have found little evidence of significant DA production in non-nutrient limiting conditions. Domoic acid is produced under conditions of nutrient stress which typically occurs when the nutrient ratio is sufficiently skewed that one nutrient becomes limiting for growth.

Pan et al. (1996) studied the effects of Si limitation on the production of DA by *Pseudo-nitzschia multiseriata*. DA was produced when population growth rate was declining and was at a maximum when Si was depleted. These workers suggested their culture data were consistent with field observations made during the first DA incident in Prince Edward Island, Canada. On that occasion, toxin production was evident 10 days after the peak of the bloom, when Si in the water was depleted. These results are supported by Fehling et al. (2004) who found greatest toxin production per cell in the Si limited stationary phase of *Pseudo-nitzschia seriata* growth. Since higher concentrations of DA were produced under Si compared to P limitation, this suggests that the form of nutrient limitation is an important factor in governing toxicity (Fig. 4). Fehling et al. (2005) also identified a photoperiod effect on growth and toxicity. Wells et al. (2005) reported a link between domoic acid, iron and copper, suggesting that a complex suite of factors influence DA production.

While laboratory studies clearly link DA production to nutrient stress, field data are less clear. Marchetti et al. (2004) questioned the reason for DA production in apparently healthy growing *Pseudo-nitzschia* communities. Similarly, Fehling et al. (2006) noted that ambient nutrient ratios were indicative of the *Pseudo-nitzschia* spp. population being N rather than Si or P limited at their Scottish coastal site where DA was evident. On the basis of the laboratory studies above, substantial production of DA would not be expected.

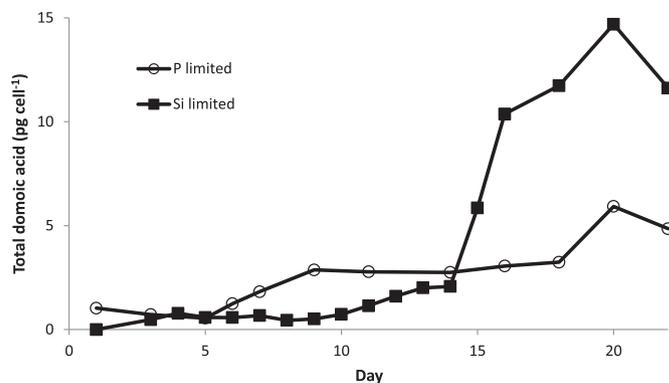


Fig. 4. Mean domoic acid concentration expressed as whole-culture domoic acid divided by cell number, in P and Si limited *Pseudo-nitzschia seriata* batch cultures. Redrawn from Fehling et al. (2004).

Fehling et al. (2006) suggested that the rapid utilisation of regenerated  $\text{NH}_4^+$  may act to alleviate N stress and hence produce the P or Si limited conditions necessary for toxin production.

PSP toxins are nitrogenous compounds and N is required for their synthesis. This suggests that N stress (i.e. a low N:P ratio) would limit toxin synthesis and a number of laboratory studies have demonstrated enhanced PSP toxin production under P stress. Murata et al. (2006) found that *Alexandrium tamarense* became more toxic at higher N:P ratios, an observation that they related to the toxin being proportional to cellular protein content. Similarly, Granéli et al. (1998) found that relative to N deficiency, P deficiency resulted in a 3-fold increase in toxin level in *A. tamarense* and presumed that toxin production was a mechanism for excess N storage. These authors also suggested that toxin production was under genetic control, but that toxin content per cell was influenced by a variety of abiotic (temperature, light, nutrient concentration) and biotic (competitors, grazers) factors.

Other studies that have linked P-stress to increased *Alexandrium* toxicity include Boyer et al. (1987); Anderson et al. (1990); Taroncher-Oldenburg et al. (1999); and John and Flynn (2000, 2002). However, this pattern is not universal. For example, Lim et al. (2010) reported increased toxicity of *Alexandrium minutum* under P stressed conditions of elevated N:P. Flynn and Flynn (1995) found toxin production rates in this species to decrease under P-stress, suggesting that P may be involved in the regulation of toxin synthesis. Flynn and Flynn (1995) also found that toxin synthesis continued after N exhaustion but then fell to very low levels. They concluded that the interpretation of toxin/cell nutrient relationships in *Alexandrium* species is complicated.

Toxicity may also be governed by the particular species or strain. For example, in Scottish waters, high toxicity Group I *Alexandrium tamarense* is thought to have dominated historically (Davidson and Bresnan, 2009) and resulted in frequent shellfish toxicity. Recently, however, *A. tamarense* blooms with little or no PSP toxicity have increased in frequency. This has been related to the presence (and presumed increasing abundance) of non-toxic group III *A. tamarense* (Collins et al., 2009; Touzet et al., 2010), rather than any variability in the toxicity of the group I strain.

*Dinophysis* species are implicated in diarrhoeic shellfish poisoning events. Species of this genus have only recently been established in long term laboratory culture, and are known to exhibit mixotrophy. The role of nutrients in their nutrition and toxin production is therefore not yet fully established. However, for *Dinophysis acuminata*, Johansson et al. (1996) found elevated okadaic acid (OA) toxin under both N and P limitation but there was a 6-fold larger enhancement in the former condition. Granéli et al. (1998) also found that the highest toxin content occurred under N limitation.

*Chrysochromulina polylepis* is a planktonic flagellate most notable for causing massive fish kills in the Kattegat in 1998. Based on semi-continuous cultures, Johansson and Granéli (1999) found its toxicity was strongly influenced by the physiological state of cells and that this provided one explanation for the large variability in the toxicity of this species. High N:P ratios resulted in increased toxin levels in *C. polylepis*, a result that is somewhat surprising since *C. polylepis* toxins are N-poor. As discussed by Granéli and Flynn (2006) and Paerl (1997), this suggests that toxicity is due to disturbed physiology under P stress.

Several authors have related changes in toxicity to the N:P ratio for other species. Flynn et al. (1996) and Granéli et al. (1998) reported an increase in PSP toxins per cell under P deficiency for *Gymnodinium catenatum*. Similar relationships were reported for the cyanobacterium *Nodularia spumigena* (Stolte et al., 2002b) and the haptophyte *Prymnesium parvum* (Shilo, 1982; Johansson and Granéli, 1999). These and other studies were reviewed by Granéli

and Flynn (2006) who found that the relationship between nutrient ratios and toxicity was genus, and in some cases, species specific.

In summary, while other factors such as bacterial interaction continue to be debated (Green et al., 2010) there is increasing evidence from algal cultures that many harmful algae become more toxic when cells are 'nutrient stressed'. That is, when one nutrient becomes limiting and nutrient supply ratios are markedly different from Redfield. The general explanation seems to be that the toxin is synthesized when biomass synthesis slows. Such findings might imply that cells are likely to become more toxic towards the end of a bloom, but this does not help to explain the suggested widespread increase in HABs or toxicity in the last few decades.

## 8. Synthesis

The nutrient ratio hypothesis that anthropogenic perturbation in the nutrient supply ratio can influence the floristic composition of phytoplankton is well established, with the influence of a change in the ratio of inorganic N:Si being particularly clear. There is, however, an important caveat: nutrient ratios are only important when the concentration of one nutrient is low enough to limit growth. Therefore, if the concentration of each nutrient contributing to the ratio is not limiting, then the value of the nutrient ratio is not important in determining species competition, and its use could be misleading.

A link between anthropogenically driven changes in nutrient ratios and an increase in the occurrence of HABs is not easy to establish. In reviewing evidence from the most cited examples we found considerable evidence to question that a direct causal link exists. In each case, our analysis highlights that the original papers are generally measured and careful in their interpretation of the strength of the control afforded by nutrient ratios, but in some cases, subsequent quoting of these articles has led to an "inflation" of these claims.

Mesocosm and field studies suggest that it is not valid to regard the Redfield ratio as a consistent tipping point from N to P or Si limitation, and also that large shifts in nutrient ratios are often needed to bring about changes in phytoplankton composition in coastal marine waters. Moreover, with the exception of some specific events, nutrient ratios in coastal waters change relatively slowly even if this change is anthropogenically driven. Hence, a problem with proving or disproving any link between nutrient ratios and the occurrence of harmful algae, rather than a simple diatom/dinoflagellate shift, is frequently the lack of a sufficiently long time series of nutrients, harmful phytoplankton, and other possibly causative environmental variables.

In European waters, the occurrence (where none have occurred before), increase in frequency or spatial and temporal extent of HABs is one indicator of the undesirable disturbance that can be associated with eutrophication (EU Urban Waste Water Treatment Directive, Council Directive 91/271/EEC). There is therefore an interest in winter concentrations of dissolved inorganic nutrients and ratios by policy makers concerned with minimising the effects of anthropogenic nutrient enrichment. For example, the OSPAR comprehensive procedure to combat eutrophication includes winter dissolved inorganic nutrient ratios as one of several indicators of anthropogenic nutrient enrichment. In addition, the eutrophication task group commissioned by the EU to develop guidance on the implementation of the Marine Strategy Framework Directive also suggested the use of perturbations in winter N:P ratios as an indicator of eutrophication potential (Ferreira et al., 2011). The findings of this review show that there is little evidence that shifts in inorganic N:P ratios promote the occurrence of HABs and this raises doubt over using such ratios in assessing the

risk of coastal eutrophication or that nutrient reduction strategies, that purport to 'better' balance the nutrient ratio, will necessarily have the desired effect of reducing the frequency and magnitude of HABs. Rather, we concur with Flynn (2010) that [summer] nutrient concentrations rather than ratios (at least for N and P) are most likely to govern phytoplankton abundance, although not necessarily to promote HABs (Gowen et al., 2012).

While the potential negative health impacts of HABs are clear, they are poorly quantified (Hinder et al., 2011) and frequently mitigated by monitoring programmes. Similarly, economic impacts of HABs remain poorly understood (Hoagland and Scatasta, 2006). Hence, even if remediation of summer nutrient ratios could be achieved in a way that reduced HABs, it is unclear how the necessary cost/benefit analysis of the undoubtedly major financial investment that would be required to achieve this could be carried out.

There is substantial and growing evidence that some HAB species are active mixotrophs with the ability to utilise DOM and/or ingest particles. It is clear that considerable further study is required to quantify the importance of mixotrophy in the development of HABs. Comparative studies to determine if HAB species are more capable of utilising dissolved and particulate organic nutrition compared to other benign species are lacking, as is an understanding of how the relative availability of inorganic and organic nutrients influences the growth of mixotrophic species. Collos et al. (2009) also pointed out that the traditional use of molar N:P ratios of dissolved inorganic nutrients cannot be used for species that are not obligate autotrophs. This excludes mixotrophic species and many dinoflagellate HAB species are mixotrophs, and this raises further doubt over the use of inorganic N:P ratios as an indicator for the probable occurrence of HABs (and eutrophication).

Uncertainty regarding the magnitude and composition of the labile DOM pool in coastal waters precludes any easy determination of the role of DOM in stimulating HABs. Better analytical quantification of the DOM pool is required, together with an understanding of which, if any, fractions of the DOM promote HABs. Within the DOM pool, urea is of particular concern because of its increased use as an N fertilizer for agriculture. As yet, evidence of a clear link between HABs and anthropogenic urea is lacking in all but a few localised environments. While some studies have been undertaken (e.g. Fan et al., 2003), comparative studies of the importance of urea as a substrate for HAB and non-HAB species under conditions in which blooms are most likely to occur are also urgently required. In particular, there is a need for mesocosm and field scale experiments (most likely using stable isotopes) to determine whether N supplied as urea rather than inorganic forms promotes the growth of HAB species. However, field based methods to determine nutrient utilisation by low biomass biotoxin producing species remains an important and unresolved challenge for urea, as well as other forms of nutrient. Molecular methods, such as the determination of gene expression may, with time provide the tools to address such problems. Given the increasing importance of coastal aquaculture as a human food source in many parts of the world (Anderson and Wolff, 2005) if runoff of agriculturally derived urea is shown to have a widespread influence on HAB development, it will generate a complex debate, with potential conflict between the need to use urea in intensive terrestrial agriculture and the possible impact of HABs on coastal aquaculture.

Nutrient ratios have most clearly been linked to biotoxin production by some key HAB species. Major PSP and ASP toxins such as saxitoxin and domoic acid respectively are N-containing and hence cannot be synthesised in its absence. Culture studies using different species of *Pseudo-nitzschia* offer particularly convincing evidence of toxicity being generated in (non-N) nutrient limited post exponential phase of growth. However, there is a marked difference between the precise control of algal growth by

nutrients in culture and conditions in the sea where multiple stressors, that may influence toxicity, exist. Hence translation of even these laboratory findings to the field becomes problematic with toxicity being observed in situations when, on the basis of bulk nutrient ratios, it would not be expected. This may be related to our inexact definition of nutrient ratios and suggest that more rigour is required to quantify the role of different inorganic and organic nutrient pools during harmful blooms. However, it is clear that further studies are also required to tease out the influence of nutrient cycling and limitation by micro (e.g. iron) nutrients and how these relate to HAB toxicity.

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