Vol. 75: 259–269, 2015
doi: 10.3354/ame01762

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online July 20

Cellular nutrient content measured with the nuclear microprobe and toxins produced by *Dinophysis norvegica* (Dinophyceae) from the Trondheim fjord (Norway)

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ABSTRACT: *Dinophysis norvegica* is a commonly occurring dinoflagellate species and a producer of diarrhetic shellfish poisons. Natural samples were collected from Trondheim fjord, Norway, to analyse nutrient (O, C, N, P) and toxin (dinophysitoxins [DXTs], okadaic acid [OA], pectenotoxins [PTXs]) content in *D. norvegica* cells. Nutrient and toxin analyses were also carried out on cells grown under nutrient-sufficient and nutrient-deficient conditions to determine how intracellular nutrient and toxin content varied under different nutrient availability conditions. Nutrient analyses were conducted using nuclear microprobe techniques that can accurately analyse single cells, and toxin analyses were carried out using liquid chromatography and mass spectroscopy. The intracellular carbon, nitrogen and phosphorus content in individual cells varied greatly, and intracellular C:N:P ratios showed that the cells were both N- and P-deficient when compared to the Redfield ratio. The ideal N:P ratio in the media for *D. norvegica* was found to be below the Redfield ratio, but intracellular ratios did not show a clear relationship with those in the media. N:P ratios of *D. norvegica* were higher than expected, which is likely due to their phagotrophy on zooplankton. The highest toxin values found were traces of PTX2, 24.72 pg PTX2SA cell^{-1}, 2.19 pg DTX1 cell^{-1}, and 1.01 pg OA cell^{-1}. However, we found no clear relationship between the content of intracellular nutrients and toxins.

KEY WORDS: *Dinophysis* · Diarrhetic shellfish poisoning · Elemental cellular content · Mixotrophy · Nuclear microprobe · Nutrient ratios · Nutrients · Trondheim fjord

INTRODUCTION

The dinoflagellate *Dinophysis norvegica* Claparède and Lachmann, 1859 is a commonly occurring species in the North Sea, where it has been described as an early summer species, although it is also present during the autumn months (Dodge & Hart-Jones 1974). On the southern coast of Norway, *D. norvegica* is abundant from March until December (Naustvoll et al. 2012) as well as in the Sognefjord on the Norwegian West coast, where it is most abundant in the autumn together with *Dinophysis acuta* (Séchet et al. 1990).

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D. norvegica, like many other *Dinophysis* species, produces diarrhetic shellfish poisoning (DSP) and are responsible for toxicity events worldwide (Hallegraeff 1993). Specifically, *D. norvegica* causes toxicity events in Norway (Séchet et al. 1990), Canada (Subba Rao & Pan 1993) and the Baltic Sea (Setälä et al. 2011). The most common DSP toxins include okadaic acid (OA) and dinophysitoxins (DXTs), with DTX1 being often detected (Yasumoto & Murata 1993). *Dinophysis* species also produce pectenotoxins (PTXs), which are not associated with DSP symptoms (Draisci et al. 1996). PTX2 is a common PTX that is later metabolised, with pectenotoxin-2 seco acid (PTX2SA) as a primary product. *Dinophysis* toxin profiles from different geographical isolates have been shown to vary, even when grown under the same environmental conditions (Fux et al. 2011). For example, *D. acuta* accumulated toxins in the cells when starved of prey, but toxin production declined with growth rates (Nielsen et al. 2013). Previous studies also found that toxin content in *Dinophysis acuminata* reached its maximum in the mid-stationary phase when subjected to various light treatments (Tong et al. 2011). The toxicity of *Dinophysis* may also increase under stress conditions such as inorganic nutrient limitation (Johansson et al. 1996), which has also been observed in other dinoflagellate species (Boyer et al. 1987). However, recent studies with *Prorocentrum lima* have also described toxin accumulation due to the slowed growth rate under nutrient-limiting conditions (Varkitzi et al. 2010). These nutrient studies considered the nutrient concentration in the media, yet, different species, comprising different biochemical compositions have different nutrient requirements (Harrison et al. 1990). Thus, the internal nutrient status should be a more reliable measurement of nutrient sufficiency or deficiency (Droop 1974, Holm & Armstrong 1981). These measurements are usually compared with the Redfield ratio of 106:16:1 (carbon [C]: nitrogen [N]: phosphorus [P], by atoms) for mixed phytoplankton growing in the open ocean (Redfield et al. 1963). Species-specific ratios may, however, vary significantly (Sakshaug & Olsen 1986), with the Redfield ratio being the average of species-specific ratios (Klausmeier et al. 2004). Moreover, *D. norvegica* is an obligate mixotroph (Park et al. 2006), taking up carbon via both photosysntethis and phagotrophy (Granéli et al. 1997b, Gisselson et al. 2002).

Nuclear microprobe (NMP) techniques can be used to measure the nutrient status in individual cells. NMP techniques use proton beams which are long enough to analyse the whole cell compartment (Moretto & Llabador 1997) and allow multiple analyses to be carried out simultaneously (Tapper & Malmqvist 1991), which constitute an advantage over electron microanalysis that is currently often used for elemental analysis in phytoplankton cells (Clay et al. 1991, Sigee et al. 1998, Rengefors et al. 1999). Whilst electrons are most suitable for detecting heavier elements (Llabador & Moretto 1998), protons can also detect light elements with detection limits usually 2–3 orders of magnitude greater than those of the electron microprobe (Tapper & Malmqvist 1991). The NMP techniques have also previously been used in the study of phytoplankton cells (Gisselson et al. 2001, Pérez-Blanco et al. 2013), and the principles of the NMP techniques are given in Tapper & Malmqvist (1991), and Llabador & Moretto (1998).

In this study, NMP techniques were used to estimate the intracellular composition of C, N, oxygen (O) and P in single *D. norvegica* cells. The aim of this study was to investigate the nutrient content of single *D. norvegica* cells in the natural environment using NMP, to understand the nutrient status in the natural environment and its variability within the population. Cells were experimentally grown under nutrient-sufficient and nutrient-deficient conditions to determine how intracellular nutrient and toxin contents varied according to nutrient availability, and to identify potential relationships between cellular nutrient content and toxin content.

MATERIALS AND METHODS

Sampling and sample processing

Natural samples were collected in the Trondheim Fjord, Norway, on 26, 30, 31 August, and 1 September 1999. Cells were collected and concentrated in a 20 µm mesh plankton net from a depth of 15 m. Cells were further concentrated by filtration through a 60 µm mesh net and onto a 40 µm mesh net. Cell concentrates were kept in filtered seawater for cell isolation or further concentration on a 20 µm mesh net and conserved using methods depending on their subsequent use. Cell isolation was carried out using a micropipette under an inverted microscope (Nikon Diaphot) and cells were transferred twice in filtered seawater (Whatman GF/F) to remove other phytoplankton species.

Filtered seawater from samples collected between 30 August to 1 September was directly analysed for inorganic nutrient concentrations (NO_3 and PO_4) using a Technicon auto-analyser at the Trondheim

biological station (NTNU) after adding 0.5 ml H_2SO_4 4M to 50 ml of filtered water. Samples for cell counts were fixed with Lugol's iodine solution and were counted on an inverted microscope according to the Utermöhl method (Utermöhl 1958). Samples collected on 30 August were lost during transportation and therefore no data on the phytoplankton community exist for this date.

Two replicates of each sample were preserved in ethanol (final concentration 70%) for toxin analyses. Each sample contained ca. 1000 previously isolated cells. Samples were dried by evaporation and dissolved in methanol-water (9:1, v/v) for liquid chromatography/mass spectroscopy (LC/MS) analysis carried out according to Goto et al. (2001). For this, a chloroform extraction was carried out, followed by a clean-up and chromatographic separation and quantification with reference toxins.

The samples for element content analysis contained ca. 300 previously isolated cells and were prepared according to Pérez Blanco et al. (2013). Once the samples had been filtered through a 20 µm mesh net, they were rinsed twice with 30 ml ice-cold Milli-Q water to remove salt (no vacuum was applied during filtrations since it could potentially interfere with the NMP techniques). The cells on the net were then transferred to a plastic film (Kimfol) by pressing the net to the film. The samples on the Kimfol were immediately stored at −20°C, and later freeze-dried (Christ Alpha 2-4). Freeze-dried samples were stored in a desiccator until the NMP analysis was performed. The nuclear microprobe was calibrated as in Pallon et al. (1999). Intact cells on the Kimfol were picked up under the microscope to be scanned by the NMP for measurements of C, N, P and O (data for O not shown) and results converted to units of pmol cell^{-1}. Specifically, P was quantified using particle-induced X-ray emission (PIXE), and C, N and O was quantified using proton backscattering spectrometry (BS). The mass density (mg cm^{-2}) of the sample was estimated using scanning transmission ion microscopy (STIM) (Pallon et al. 2004). Scans were carried out according to Gisselson et al. (2001) to properly select the area scanned as well as to obtain blanks for background corrections. Although efforts were made to freeze dry cells as soon as possible after the rinsing step, some cells were damaged. Therefore, the number of cells analysed from each sampling date varied: 8, 14, 6, and 12 cells were analysed from samples from 26, 30, 31 August and 1 September, respectively.

Nutrient experiments

On 31 August, an extra sample containing a population of *D. norvegica* was concentrated using methods described in 'Sampling and sample processing' section above. The phytoplankton concentrate from the 20 µm mesh net was diluted with Whatman GF/F filtered seawater and poured into a 600 ml Erlenmeyer flask. The filtered seawater had initial concentrations of 0.43 µM NO_3^- and 0.12 µM PO_4^{2-}. This sample was thereafter divided into 3 Erlenmeyer flasks to which nitrogen or phosphorus were added separately (giving N- or P-deficient treatments), or in combination for the nutrient-sufficient treatment. The final nitrogen and phosphorus concentrations for each treatment are shown in Table 1, and all had a final volume of 200 ml. The flasks were then incubated in a culture room at 15°C on a 15 h light:9 h dark cycle (irradiance = 80 µmol m^{-2} s^{-1}) for 5 d. These conditions were similar to those *in situ* regarding the temperature, although light intensity was higher than under the natural conditions for the cells collected at the lower depths.

Following incubation, the cultures were again filtered to obtain cell samples for counts, and for toxin and NMP analyses, which were conducted using methods identical to those used for the natural samples (see 'Sampling and sample processing' section above for details on sample preparation and analyses) . The number of cells available for NMP was also variable (16 cells for nutrient-sufficient and P-deficient treatments, and 17 cells for N-deficient treatments).

Statistical analyses

Statistical analyses were carried out using SPSS 20.0. Statistical differences between treatment groups

Table 1. Inorganic nutrient content in the water in natural samples (collected on 26, 30, 31 August and 1 September) and in the media for the experimental nutrient cultures (NP-sufficient, N-deficient, P-deficient) and % *Dinophysis norvegica* cells with respect to the total of species present

Sample	Nutrients (µM)			*D. norvegica* cells (%)
	NO_3^-	PO_4^{3-}	N:P	
All water samples	–	–	–	–
26 August	–	–	–	1.6
30 August	0.14	0.06	2.33	–
31 August	9.70	0.71	13.66	14
1 September	11.40	0.81	14.07	24
NP-sufficient	32.43	5.12	6.33	76
N-deficient	0.43	5.12	0.08	71
P-deficient	32.43	0.12	270.25	67

were calculated using the Kruskal-Wallis test and the Mann-Whitney *U*-test. The different parameters were also checked for correlations (Spearman's rank). For all analyses the p-value was set at 0.05 unless otherwise stated.

RESULTS

Inorganic nutrients and phytoplankton species

Nutrient concentrations (N and P) in the seawater increased over the sampling period (Table 1). Samples contained *Dinophysis norvegica* as well as other dinoflagellate species such as *Dinophysis acuminata, Ceratium* spp., *Protoperidinium* spp., and *Prorocentrum micans*, which was only found in the sample collected on 1 September. The samples also contained various species of pennate diatoms, which constituted ca. 80% of the phytoplankton community during the first days of sampling, and decreased to about 50% on the last sampling day. The percentage of *D. norvegica* relative to the whole phytoplankton community increased over the sampling period (Table 1).

In the nutrient experiments, cultures initially contained *D. norvegica* densities of 82 cells ml^{-1}. At the end of the experiments, cell densities of *D. norvegica* had increased and comprised similar proportions of total phytoplankton population in all nutrient conditions (Table 1). The estimated specific growth rates were 0.24 d^{-1} in the nutrient-sufficient culture, and 0.13 d^{-1} and 0.14 d^{-1} in the N- and P-deficient cultures, respectively.

Nuclear microprobe analyses of *Dinophysis norvegica*

The average area of cells, intracellular C, N and P content as well as their ratios from both natural samples and the nutrient experiments are summarised in Table 2. All nutrient ratios were high compared to the Redfield ratio, indicating that cells were both N- and P-starved. The variability in the intracellular nutrient content of different cells (Fig. 1) was largely reflected in the nutrient ratios (Table 2); therefore, we considered nutrient ratios of the mean nutrient content as a more realistic approach to the intracellular ratios than the mean of the ratios, which were very high in some cases. Nevertheless, in the natural samples, the distributions of C, N and P as well as the C:N, N:P and C:P ratios were the same among the sampling dates ($p < 0.05$); consequently, all cells

Table 2. Average (±SD) area, nutrient (C, N and P) content and nutrient ratios of *Dinophysis norvegica* cells analysed using nuclear microprobe techniques for natural samples (collected on 26, 30, 31 August and 1 September) and after nutrient treatments (NP-sufficient, N-deficient, P-deficient)

	Area (μm^2)	Nutrients (pmol cell^{-1})					
		C	N	P	C:N	C:P	N:P
All water samples	2649.36 ± 624.92 (n = 40)	487.12 ± 225.38 (n = 40)	30.92 ± 17.37 (n = 40)	0.68 ± 0.60 (n = 40)	17.72 ± 7.36 15.75[a]	2279.35 ± 4712.81 711.78[a]	137.49 ± 327.01 45.16[a]
26 August	2947.62 ± 377.58 (n = 8)	703.99 ± 302.55 (n = 8)	39.8 ± 23.2 (n = 8)	0.49 ± 0.36 (n = 8)	19.75 ± 7.89 17.70[a]	3380 ± 4206 1436.30[a]	172.38 ± 188 81.15[a]
30 August	2717.17 ± 589.65 (n = 14)	434.60 ± 129.01 (n = 14)	27.04 ± 17.18 (n = 14)	0.67 ± 0.70 (n = 14)	19.96 ± 8.56 16.07[a]	3543.49 ± 7161.55 650.25[a]	222.32 ± 531.87 40.45[a]
31 August	2575.45 ± 729.16 (n = 6)	372.55 ± 183.50 (n = 6)	31.59 ± 19.63 (n = 6)	0.89 ± 0.56 (n = 6)	12.76 ± 2.29 11.79[a]	511.82 ± 208.77 416.51[a]	42.02 ± 19.83 35.32[a]
1 September	2408.36 ± 710.21 (n = 12)	461.10 ± 203.09 (n = 12)	29.22 ± 11.10 (n = 12)	0.73 ± 0.65 (n = 12)	16.22 ± 6.16 15.78[a]	954.31 ± 565.68 633.84[a]	63.01 ± 32.69 40.17[a]
NP-sufficient	2769.68 ± 594.11 (n = 16)	526.20 ± 250.66 (n = 16)	41.88 ± 21.82 (n = 16)	1.23 ± 0.54 (n = 16)	15.03 ± 9.86 12.56[a]	541.31 ± 417.34 426.63[a]	42.26 ± 30.85 33.96[a]
N-deficient	2472.24 ± 499.04 (n = 17)	656.45 ± 307.49 (n = 17)	44.54 ± 24.60 (n = 17)	0.80 ± 0.61 (n = 17)	16.74 ± 8.87 14.74[a]	2206.14 ± 2913.08 817.20[a]	126.56 ± 173.35 55.44[a]
P-deficient	1976.84 ± 661.14 (n = 16)	205.64 ± 119.52 (n = 16)	12.86 ± 6.53 (n = 16)	0.11 ± 0.15 (n = 16)	18.52 ± 11.32 16.00[a]	13482.05 ± 21479.51 1957.11[a]	838.18 ± 1515.27 122.35[a]

[a]Ratios calculated as ratios of mean elemental contents per cell instead of the mean of elemental ratios

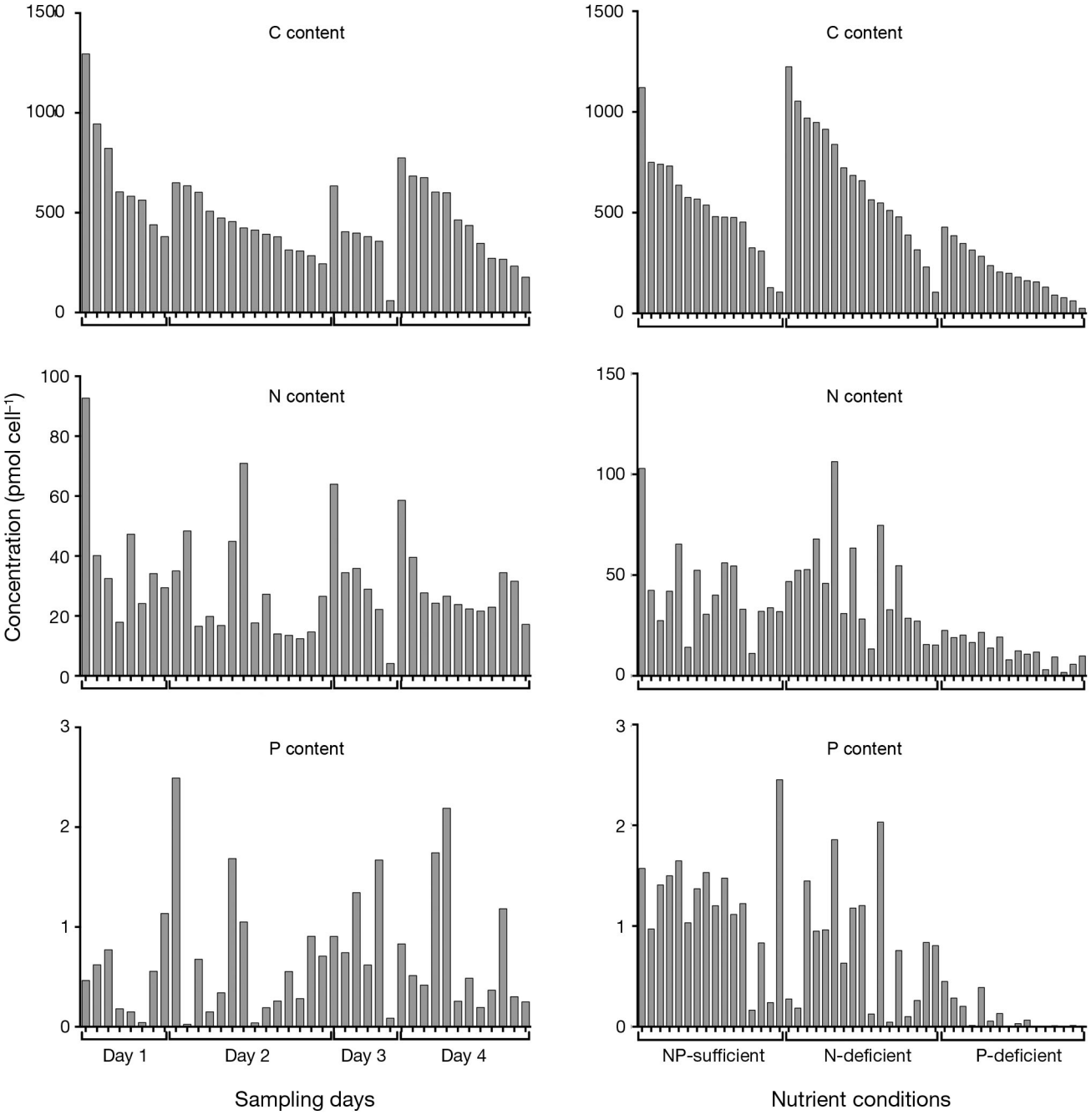

Fig. 1. C, N and P content per cell analysed using nuclear microprobe (NMP) techniques in natural samples (sampling days 1–4, collected on 26, 30, 31 August, and 1 September, respectively) and in each of the experimental culture conditions (NP-sufficient, N-deficient, P-deficient). Each column represents a single cell

were pooled together for further analyses and comparisons with the cells in culture (Fig. 2). The cells from all water samples together were divided into 2 groups (high and low average nutrient ratios), which revealed that P content was the main cause of variability in both C:P ($U = 14$, $p < 0.001$) and N:P ratios ($U = 16$, $p < 0.001$). Likewise, the C:N ratios varied due to the N content ($U = 82$, $p < 0.005$) and in this case, differences in P content were not observed among cells with high and low C:N ratios.

In the P-deficient culture, all elemental content (including O [data not shown]) was lower than in the other treatments ($p < 0.05$), and P-deficiency was clearly reflected in the higher C:P and N:P ratios ($p <$

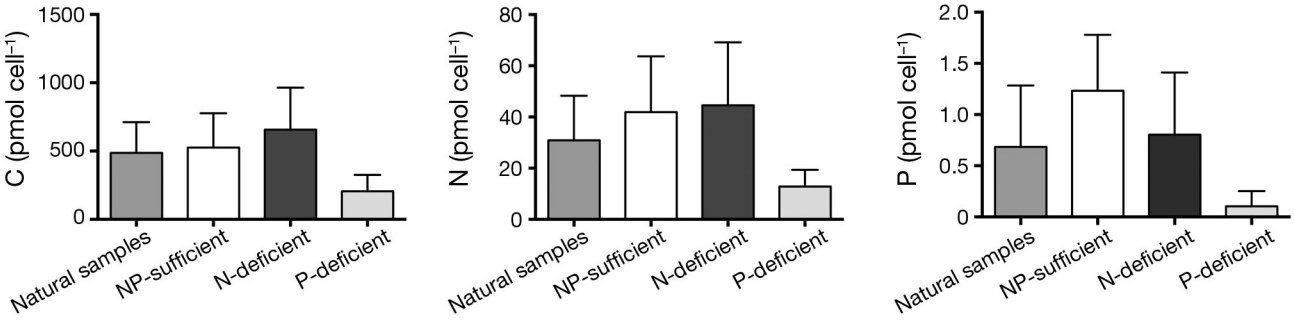

Fig. 2. Average (±SD) C, N, and P content per cell from natural samples (sampling days 1–4, collected on 26, 30, 31 August, and 1 September, respectively) and each of the experimental culture conditions (NP-sufficient, N-deficient, P-deficient) analysed using nuclear microprobe techniques

0.05). The N-deficient culture showed a higher C:N ratio than the nutrient-sufficient culture. Although the C:P and N:P ratios of the N-deficient culture were not as high as those observed in the P-deficient culture, their high values made it seem P-deficient. When comparing natural samples with the culture samples, statistical differences in C, N, P, and O content were also found in the P-deficient culture ($p < 0.05$).

The area of the cells (Table 2) in the natural samples ranged from 1156–3721 μm^2, whilst in the experiment cultures this range was much greater (567–4169 μm^2), with smaller cells in the P-deficient culture and larger cells in the nutrient-sufficient culture ($p < 0.05$). The area of the cells in the P-deficient culture also differed statistically from the area of the cells in the natural samples ($p < 0.05$).

In the water samples, the cellular content of C and O (data for O not shown) was significantly correlated with the area of the cells ($p < 0.01$), but the N and P content was not correlated with cell area ($p < 0.05$). In the nutrient experiments all elemental content correlated with the area of the cells ($p < 0.01$).

In water samples, there was a significant correlation between PTX2SA and both C and P content ($p < 0.01$), which was reflected in the C:P and N:P ratios ($p < 0.01$). The cells in the experiment cultures showed significant correlations between PTX2SA and C, N and O content, but not with P content ($p < 0.01$). DTX1 and OA had a significant correlation with the P content as well as with the C:N, C:P and N:P ratios. When considering all natural and experimental culture samples together, the only significant correlations found were between PTX2SA and the C ($p < 0.01$) and O ($p < 0.05$) contents.

DISCUSSION

Growth rates for some *Dinophysis* species have been estimated using different methodologies, under different environmental conditions and reported in various ways (Table 4). In some cases, experiments have been carried out to simply determine a species growth rate (e.g. Chang & Carpenter 1991), whilst others have tested the effects of various factors such

Toxin analyses

Pectenotoxins (PTX2, PTX2SA), dinophysitoxins (DXT1), and okadaic acid (OA) were found in *D. norvegica* cells both from the field samples and from the nutrient experiments (Table 3). Toxin content was similar during the 4 sampling dates, although an increase in DTX1 was observed during the last day. Furthermore, after incubation with different amounts of N and P available, DTX1 and OA had increased in all treatments.

Table 3. Average (±SD) toxin content in *Dinophysis norvegica* for natural samples (collected on 26, 30, 31 August, and 1 September) and after nutrient treatments (NP-sufficient, N-deficient, P-deficient). nd = not detected

Sample	Toxins (pg cell^{-1})			
	PTX2	PTX2SA	DTX1	OA
All water samples	–	21.63 ± 3.61	–	0.21 ± 0.08
26 August	Traces	24.72 ± 1.66	Traces	0.28 ± 0.03
30 August	Traces	23.54 ± 0.15	nd	0.14 ± 0.04
31 August	Traces	16.53 ± 0.16	nd	0.28 ± 0.09
1 September	nd	21.74 ± 5.45	2.19 ± 0.15	0.16 ± 0.10
NP-sufficient	Traces	22.03 ± 1.63	1.60 ± 0.16	1.01 ± 0.34
N-deficient	Traces	24.02 ± 0.31	2.00 ± 0.75	0.71 ± 0.13
P-deficient	nd	19.53 ± 4.03	2.13 ± 0.35	0.74 ± 0.49

Table 4. Estimated growth rates of *Dinophysis* species and methodologies used derived from the current literature

Species	Origin	Method	Growth values	Based on	Source
D. norvegica	Baltic Sea	Growth rate (d^{-1})	0.1–0.4	Cell cycle analysis	Gisselson et al. (2002)
D. acuminata	Woods Hole, NE, USA	Growth rate (d^{-1})	0.12–0.21	Cell counts	Tong et al. (2011)
D. norvegica	E Skagerrak, Sweden	Growth rate (d^{-1})	0.18, 0.29 and 0.61	C-uptake	Granéli et al. (1997a)
D. norvegica	Skagerrak, Sweden	Doublings d^{-1}	0.25–0.38	C-uptake	Granéli et al. (1995)
D. acuminata	Masan Bay, Korea	Growth rate (d^{-1})	0.31–0.95	Cell counts	Park et al. (2006)
D. acuta	E Skagerrak, Sweden	Growth rate (d^{-1})	0.35 and 0.41	C-uptake	Granéli et al. (1997a)
D. acuta	S France	Doublings d^{-1}	0.36–0.45	C-uptake	Granéli et al. (1995)
D. acuminata	E Skagerrak, Sweden	Growth rate (d^{-1})	0.49 and 0.59	C-uptake	Granéli et al. (1997a)
D. acuminata	Long Island Sound, USA	Growth rate (d^{-1})	0.54 and 0.67	Cell cycle analysis	Chang & Carpenter (1991)
D. acuminata	Skagerrak, Sweden	Doublings d^{-1}	0.52–0.73	C-uptake	Granéli et al. (1995)
D. acuta	Portugal	Divisions d^{-1}	0.58	Cell counts	Sampayo (1993)
D. acuminata	Portugal	Divisions d^{-1}	0.61	Cell counts	Sampayo (1993)

as light and temperature (Tong et al. 2011), light vs. dark (Granéli et al. 1997a), or type of prey available (Park et al. 2006). These studies reported growth rates from 0.1 to 1 d^{-1} for *Dinophysis acuminata*, and lower growth rates for *Dinophysis norvegica* than *D. acuminata* or *Dinophysis acuta.* This study confirmed lower growth rates in *D. norvegica* (0.24 d^{-1} in the NP-sufficient treatment) in comparison to those species, which correspond to values of 0.18 and 0.29 d^{-1} (Granéli et al. 1997a) or 0.1–0.4 d^{-1} (Gisselson et al. 2002). The lower growth rates corresponded to the nutrient-deficient cultures, although a lower rate might have been expected in the P-deficient culture than in the N-deficient culture based on the higher intracellular nutrient ratios.

The nutrient content in the water during the sampling period increased together with the N:P ratio, although, in all cases the N:P ratios were indicative of N-limitation according to the Redfield ratio (N:P < 16). However, growth of *D. norvegica* was observed, and as nutrients increased, the N:P ratio increased to ~14, therefore approaching levels of nutrient-sufficiency. Improved nutrient conditions may also have enhanced *D. norvegica* growth by increasing the availability of prey (increase in ciliates due to an increase in cryptophytes). However, it is clear that the optimal nutrient composition under nutrient-sufficient conditions is species-specific, and knowledge of these nutrient requirements is necessary to determine when nutrients are limiting growth when checking the growth media (Harrison et al. 1990). Furthermore, the N:P ratio of our nutrient-sufficient culture was low compared to the Redfield ratio (N:P = 6.33), but growth was nonetheless sustained on the nutrients available. This ratio would indicate, in the case of non-deficiency, that there was a greater excess of P rather than a deficiency of N. However, this seems unlikely considering that the intracellular N:P ratios were high in all cases.

The average C-content found in the *in situ* growing *D. norvegica* cells during this study (487.12 pmol $cell^{-1}$) (Table 2) was higher than the 400 pmol $cell^{-1}$ measured in the Baltic Sea using NMP by Gisselson et al. (2001) or the 300 or 188 pmol $cell^{-1}$ calculated by Carpenter et al. (1995) and Edler (1977), respectively. The first 2 cases can be readily explained by the cells in this study being larger (~2600 μm^2) than those in Gisselson et al. (2001), where cell areas ranged between 1200 and 2200 μm^2, with a calculated volume = 4.8×10^4 μm^3. Even smaller cells (4.33×10^4 μm^3) were observed in Carpenter et al. (1995). However, in the study by Edler (1977), cells were as large as those observed in this study and comprised a much lower estimated C content, although this may partially be due to the stoichiometric formula used to calculate those values.

In this study, nitrogen content was also higher than that of 18.5 pmol $cell^{-1}$ found by Gisselson et al. (2001), but the phosphorus content they found was higher (1.11 pmol $cell^{-1}$), which would indicate that the cells in the present study were more P-depleted; this was confirmed by the much higher C:P and N:P ratios observed here. Thus the N:P ratios found when looking at the cellular content were always higher than 16, in contrast to those in the water, and therefore indicating P-deficiency. This is not in agreement with the ratios observed in the water or with the general belief that dinoflagellates are P-limited in fresh-

water and N-limited in estuarine and marine waters (Lomas & Glibert 2000), even though co-limitation has also been described (Arrigo 2005). In the case of our study, growth was maintained with the N and P available in the media, and possibly also by phagotrophy. Optimal nutrient ratios for phytoplankton seem to range from 5 to 19 for N:P, 3 to 17 for C:N and 27 to 135 for C:P, with transition between N- and P-limitation when N:P = 20–50 (Geider & La Roche 2002). Considering these values and the fact that the 3rd sampling day had the closer ratios to the nutrient-sufficient culture, we can speculate that cells should have been in nutrient-sufficient conditions for growth, and changed towards deficiency on the 4th sampling day due to photosynthetic growth. However, the nutrient contents varied considerably among cells regardless of the sample day, and clear differences were only found within the P-deficient culture. More importantly, nutrient content in zooplankton cells shows ratios higher than the Redfield ratio (Elser & Hassett 1994). *D. norvegica* should be expected to have N:P well above 16 both because it is a phagotroph and because its prey, the ciliate *Mesodinium rubrum*, would already have a higher N:P ratio than the phagotroph itself.

According to the intracellular C:N and C:P ratios in this study, cells were both N- and P-deficient. *D. norvegica* cells from the Baltic Sea (Gisselson et al. 2001) were also N- and P-depleted, although in the present study the P-deficiency was more accentuated. This could imply that the cells had spent their P-stores, which would explain the abnormally high C:P and N:P ratios and the fact that when P was added to the media they still seemed P-deficient. This could also imply that *D. norvegica* did not have prey to feed on and that they used photosynthesis as their major source for growth, since the lack of prey seems to be a typical factor that limits growth in species from this genus in the field (Kim et al. 2008). High C:P and N:P ratios have also been found in cyanobacteria species (Bertilsson et al. 2003) and diatoms (Hessen et al. 2008) growing under P-depleted conditions.

The discrepancy between nutrient content in the water and in the cells was already observed in *Dinophysis* spp. by Delmas et al. (1992), who found no relationship between growth and nutrient availability. Likewise, Johansson et al. (1996) did not find a clear relationship between nutrient concentration in the media and cell growth of *D. acuminata* and *D. acuta.* All these discrepancies between ratios in the cells and in the environment could be at least partially explained by the influence of phagotrophy, increasing nutrients in the cells in comparison to those which would be expected from inorganic nutrient sources and changing their proportions.

For the *in situ* samples, *M. rubrum,* the prey of *Dinophysis* species, would likely have been present during sampling, and in that case it could have been at least partially retained in the filtrates used to prepare the cultures. However, this would not be the case of *M. rubrum* prey (small algae), which would have been washed out of the samples. Nonetheless, considering the length of the experiments, growth for both species could be sustained by photosynthesis (Park et al. 2008, Hansen et al. 2013). Furthermore, low N or P in the water induced phagotrophy in the dinoflagellate *Ceratium furca* (Smalley et al. 2003). *Dinophysis* showed high N:P ratios in all cases, both *in situ* and under cultured conditions, and even when C:N ratios showed N-deficiency. The high N:P ratios of *D. norvegica* in this study correspond to those of a truly heterotrophic organism. In fact, *D. norvegica* nutrient ratios are comparable with those of the heterotrophic dinoflagellate *Oxyrrhis marina* when fed with N-depleted (N:P = 23) and P-depleted (N:P = 141) prey (Meunier et al. 2012).

The toxin content of *D. norvegica* both *in situ* and in the experiment culture was generally low (Table 3), although our values are comparable with some of those obtained from several *Dinophysis* species in other studies (Table 5). From our results, we cannot conclude that there is a higher toxin content under nutrient sufficiency or deficiency, although OA was higher in the nutrient-sufficient culture. Conversely, *D. acuminata* produced more OA under N-deficient conditions (23 pg cell^{-1}) in comparison to 4.8 pg cell^{-1} under P-deficient and 1.1 pg cell^{-1} under nutrient-sufficient conditions. However, *D. acuta* also produced more OA under nutrient-sufficient conditions although differences were less dramatic (Johansson et al. 1996). Although there were some significant and positive correlations between some nutrients and the toxin content, our results were not sufficiently consistent to confirm a strong relationship between these parameters. This could be due to the sometimes high variability in the toxin content, for example in the P-deficient treatment, which may have hidden a potential correlation between these parameters. Nonetheless, PTX2SA was higher in cells with higher C content, which indicates that larger cells had a higher PTX2SA content.

The NMP techniques accurately measure the elemental content in phytoplankton cells, but are time consuming, expensive and limit the number of cells that can be analysed in comparison to bulk methods

Table 5. Toxin content in *Dinophysis* species derived from the current literature. In some cases, only ranges and the highest values are presented

Species	Origin	Toxins (pg cell^{-1})				Source
		DTX1	DTX2	PTX2	OA	
D. norvegica	Bedford Basin, Canada				0.07–54.8	Subba Rao & Pan (1993)
D. fortii	Adriatic Sea				15	Draisci et al. (1996)
D. acuminata	NW Spain		5.7		5.8	Blanco et al. (1995)
D. acuminata	Woods Hole, NE, USA	0.45–0.64		10.4–11	0.39–0.44	Smith et al. (2012)
D. norvegica	Arendal, Norway	2.5			0.8	Lee et al. (1989)
D. norvegica	Sogndal, Norway	14				Lee et al. (1989)
D. acuminata	Woods Hole, NE, USA	0.06–0.32		8.4–15.16	0.007–0.04	Tong et al. (2011)
D. acuta	W Sweden	7.0			14.0	Johansson et al. (1996)
D. acuminata	W Sweden	0.2			23.0	Johansson et al. (1996)
D. acuminata	Le Havre, France				1.6	Lee et al. (1989)
D. acuta	County Cork, Ireland		11	7.2	7	Puente et al. (2004)

(Pérez-Blanco et al. 2013). Nevertheless, NMP provides valuable information such as the variability in nutrient content within the population, while their average values correspond to those which would be obtained when using bulk methods. Pérez-Blanco et al. (2013) confirmed the validity of the average nutrient content measured by NMP with 3 species of cyanobacteria. For *D. norvegica*, this type of measurement did not match bulk methods too closely (Gisselson et al. 2001), although these results were obtained from natural samples and therefore bulk methods could have overestimated nutrient content due to the presence of other species and detritus. At the same time, the variability in nutrient content might impede the interpretation of results, since nutrients seem heterogeneously distributed (Gisselson et al. 2001), and also given that the heterogeneity of nutrient distributions may be enhanced by phagotrophy by *D. norvegica*. Nonetheless, the NMP techniques constitute a powerful tool in the field of elemental analysis that may prove useful in understanding the dynamics in a phytoplankton population and the plasticity of nutrient content.

Acknowledgements. The authors thank their colleagues at the Department of Nuclear Physics in Lund who provided assistance with the nuclear microprobe as well as C. Esplund for her technical support. We thank the Swedish Research Council (VR) for financial support and the Brazilian National Research Council for a scholarship to E.G.

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Editorial responsibility: Hugh MacIntyre, Halifax, Nova Scotia, Canada

Submitted: October 22, 2014; Accepted: May 20, 2015
Proofs received from author(s): July 10, 2015