Harmful Algae 9 (2010) 304–311

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Unbalanced N:P ratios and nutrient stress controlling growth and toxin production of the harmful dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge

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ARTICLE INFO

Article history:
Received 27 May 2009
Received in revised form 3 December 2009
Accepted 3 December 2009

Keywords:
Prorocentrum lima
Okadaic acid
Stress
Nitrate
Ammonium
Phosphate

ABSTRACT

The responses of the benthic marine dinoflagellate *Prorocentrum lima* to nutrient stress induced by unbalanced N:P ratios were the subject of this study. Batch cultures of *P. lima* cells were grown under NP sufficient (N as nitrate and ammonium) and deficient conditions, and the cell growth and toxicity were followed for eight weeks. *P. lima* grew slowly in all nutrient conditions and net growth rates ranged from 0.11 to 0.22 divisions day^{-1}. Phosphorus (P) was taken up with high uptake rates in all treatments until the end of exponential phase and reached limitation in the P deficient cultures. Nitrogen (N) did not reach limitation in any treatment. In the cultures with nitrate as exclusive N source, uptake rates of nitrate remained high after the exponential phase, suggesting that *P. lima* cells continued to accumulate N under surplus N availability. Nitrate was slowly consumed and therefore maintained cell growth, as documented by a prolonged exponential phase and an algal biomass increasing at low rates still after seven weeks of incubation. In the cultures with ammonium as exclusive N source, ammonium was taken up with the highest N uptake rates until the end of exponential phase. However, high initial concentrations of ammonium proved to be toxic to *P. lima* cells, demonstrating growth inhibition with the lowest algal biomass and okadaic acid (OA) production among treatments. The OA production increased after the exponential phase in all nutrient conditions when cell growth slowed down, suggesting that OA production was regulated by growth limitation. The highest OA cellular content (11.27 ± 3.30 pg OA $cell^{-1}$) was found in the P deficient cultures, where P decreased to limitation after the exponential phase ($P < 0.1$ μM). We argue that the severely low P concentrations slowed down the growth rate so as to allow for a higher accumulation of OA in the *P. lima* cells that continued to produce OA at the same rate.

1. Introduction

Harmful Algal Blooms (HABs) are globally increasing complex phenomena that cause serious ecological, human health and economic problems (Glibert et al., 2005). Anthropogenic eutrophication has been considered as one of the major factors triggering HAB episodes worldwide since 1980s (Anderson et al., 2002; Granéli, 2004; Pagou, 2005; Heisler et al., 2008). In many coastal areas, anthropogenic enrichment of N and P and the induced unbalanced nutrient ratios compared to the Redfield ratio have been shown to cause shifts in natural phytoplankton assemblages. Low Si:N and Si:P ratios in many eutrophicated coastal areas have been related to shifts from diatom-dominated to harmful dinoflagellate-dominated assemblages (Smayda, 1990; Moncheva et al., 2001). Furthermore, there is some proof to date that the unbalanced N:P ratios affect the physiology of several harmful algal species, such as the growth and the amounts of toxins produced by the algal cells (Granéli et al., 1998; Granéli and Flynn, 2006).

The algal toxin okadaic acid (OA) and its analogues dinophysistoxin 1 (DTX1) and dinophysistoxin 2 (DTX2) constitute a large category of polyether compounds that cause the Diarrhetic Shellfish Poisoning (DSP) syndrome to humans (Quilliam, 2003). They are produced by dinoflagellates of the genus *Dinophysis* and *Prorocentrum*. Many *Prorocentrum* species can produce DSP toxins. The potentially toxic *P. lima* (Ehrenberg) Dodge is a benthic and epiphytic dinoflagellate, widely distributed in tropical and temperate areas (Faust and Gulledge, 2002). *P. lima* has been

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doi:10.1016/j.hal.2009.12.001

linked to rather few DSP episodes in shellfish farms so far (Lawrence et al., 2000; Levasseur et al., 2003; Foden et al., 2005), probably because of the limited number of samplings in benthic and epiphytic algal assemblages, for example during monitoring programs. However, Bauder et al. (2001) have shown that *P. lima* can be ingested by shellfish and DSP toxins can accumulate in shellfish tissues for a long time. *P. lima* has also been linked to the tropical syndrome ciguatera, as it is a frequent component of the epiphytic community in ciguatera-endemic areas (Tindall and Morton, 1998).

The information on how *P. lima* growth and toxin production are influenced by different forms of macronutrients and their availability is rather limited (Morlaix and Lassus, 1992; Tomas and Baden, 1993; McLachlan et al., 1994; Sohet et al., 1995). Morlaix and Lassus (1992) measured cell densities and toxins in *P. lima* cultures with different concentrations of nitrate and glycerophosphate once in thirty days after enrichment.

Growth rates increased with increasing nitrate up to the concentration of 880 μM, but decreased in higher concentrations. Toxin production was constantly low. Glycerophosphate and growth rates followed a non-linear relation, while toxin production was inversely proportional to growth rate. McLachlan et al. (1994) investigated the impact of low nitrate concentrations on growth and toxin production in *P. lima* cultures. Toxin production increased during stationary phase in all nitrate concentrations and was therefore related to growth. In low nitrate concentrations, toxin production was highest.

The aim of this study was to examine the responses of the toxigenic dinoflagellate *P. lima* to nutrient stress induced by unbalanced N:P ratios. In order to fill specific gaps of our knowledge on these issues, we addressed the following questions: (1) how do different forms of available nitrogen affect growth dynamics and toxin production of *P. lima*?, (2) how does nutrient stress, due to deficient nitrogen or phosphorus concentrations, affect growth dynamics and toxin production of *P. lima*?, and (3) does toxin production vary as a combined function of growth, available forms of nutrients and nutrient stress? To answer these questions, we followed *P. lima* growth and toxin production under different nutrient conditions for eight weeks of incubation. Four nutrient treatments were tested; two forms of nitrogen (nitrate and ammonium) in NP sufficient conditions, and two nutrient stress conditions as N deficient and P deficient conditions. To our knowledge, this is the first time that a number of growth parameters and toxin production were monitored synchronously in *P. lima* cultures under the specified nutrient conditions. This is also the first time that the role of ammonium as an exclusive nitrogen source versus nitrate is investigated in *P. lima* cultures. Such information could give us useful insights into bloom dynamics as they currently occur in many eutrophicated coastal areas, where either ammonium or nitrate may be the dominant form of nitrogen.

2. Materials and methods

2.1. Algal cultures

Unialgal xenic batch cultures of a *Prorocentrum lima* strain (CCAP1136/11 from the Culture Centre for Algae Protozoa, isolated from Ría de Vigo, Galicia, NW Spain) were grown in f/2 medium (Guillard, 1975), modified by adding H_2SeO_3 and reducing the $CuSO_4$ concentration (Anderson et al., 1996). Nitrate and ammonium were used as separate nitrogen (N) sources and orthophosphates were used as a phosphorus (P) source. All culture media were prepared in autoclaved 0.2 μm filtered aged natural seawater, collected from offshore waters (38 psu, pH 8 ± 0.1). All cultures were incubated in 20 °C, 16 h:8 h light:dark cycle and a light intensity of 35 μmol photons m^{-2} s^{-1}, provided by cool white fluorescent tubes.

Table 1
Initial nitrogen and phosphorus concentrations (μM) and N:P ratios in the media of *Prorocentrum lima* cultures grown under four different nutrient treatments with NP sufficient (N as nitrate and ammonium) and deficient conditions.

Treatment	Initial nutrient concentrations (molar) and N:P ratios			
	Nitrogen (μM)	Phosphorus (μM)	N:P	Medium
Sufficient $N(NO_3^-)$ $P(PO_4^{3-})$	883	36.2	24:1	f/2 (NO_3^-)
Sufficient $N(NH_4^+)$ $P(PO_4^{3-})$	883	36.2	24:1	f/2 (NH_4^+)
Deficient $N(NO_3^-)$	300	72.5	4:1	N deficient f/2
Deficient $P(PO_4^{3-})$	1450	18.1	80:1	P deficient f/2

After six months of acclimation in NP sufficient conditions, exponentially growing cells were inoculated to fresh media with the experimental nutrient conditions. Four different nutrient treatments were tested; two forms of nitrogen with (1) sufficient nitrate-NP concentrations and (2) sufficient ammonium-NP concentrations, and two nutrient stress conditions with (3) deficient nitrate-N concentrations and (4) deficient P concentrations (see Table 1 for details). Duplicate cultures corresponded to each one of the four nutrient treatments. The experimental cultures were grown in 4L capacity Nalgene® polycarbonate carboys for eight weeks (57 days). Some preliminary tests were performed in order to reassure that there was not any significant change of the media pH in the polycarbonate carboys due to autoclaving (*t*-test, $p > 0.05$). Each polycarbonate carboy contained 3 L of culture medium and was gently but thoroughly agitated by hand every 24 h.

2.2. Culture samplings

Samples for the parameters described in the following paragraphs were collected on regular intervals (every 3–4 days for first 5 weeks, then every 5 days) and always at the same time of the day. A 160 mL sample for the first two weeks and a 110 mL sample for the next six weeks were removed from the culture carboys for all the analyses described next. Each carboy was thoroughly but gently shaken prior to each sampling, in order to homogenize the cultures.

2.3. Growth parameters

A 10 mL aliquot was removed from the culture sub-sample and fixed with Lugol's solution for the determination of cell densities (cells mL^{-1}). Cell densities were determined microscopically with a Sedgewick Rafter 50S chamber under a NIKON DIAPHOT microscope (150× magnification) according to Andersen and Throndsen (2003). Growth rates were calculated according to the equation of Guillard (1973), and net growth rates from the linear regressions of cell densities vs time during exponential phase. For the determination of total chlorophyll *a* (chl *a*) concentrations (μg chl *a* L^{-1}) and intracellular chl *a* concentrations (pg chl *a* $cell^{-1}$), a 50 mL culture aliquot was gently filtered through Whatman® glass fiber filters (GF/F). The cells retained on the filters were extracted with 80% aqueous acetone overnight. Total chl *a* was determined fluorometrically with a TURNER Designs® TD-700 fluorometer, according to Holm-Hansen et al. (1965).

2.4. Nutrient conditions

Nutrient analyses were done on 100 mL GF/F filtrates of the culture aliquots. Nitrate (NO_3^-) and nitrite (NO_2^-) in the culture filtrates were analyzed according to Strickland and Parsons (1968)