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First report of toxic *Prorocentrum rhathymum* (Dinophyceae) and its pigment composition from coastal waters of the eastern Arabian Sea

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Prorocentrum rhathymum,a toxic dinoflagellate, was isolated for the first time from coastal waters of the eastern Arabian Sea. Batch cultures were grown and cell pellets were extracted in methanol for analysis of algal toxin. LCMS/MS studies exhibited presence of dinophysis toxin 1 (DTX1) with no detectable Okadaic Acid. Growth rates varied between 0.23 day^{-1}(exponential phase) and 0.05 day^{-1}(decline phase). Phytoplankton pigment studies revealed the presence of chlorophyll *a*, chlorophyll c1c2, peridinin and diadinoxanthin. The average chlorophyll *a* production was found to be 163 pg $cell^{-1}$ and ranged between 73 to 223 pg $cell^{-1}$. Peridinin, the marker pigment of dinoflagellates, had an average value of 55 pg $cell^{-1}$ and ranged between 30 – 114 pg $cell^{-1}$. Further studies to identify its molecular diversity along with toxicity are presently underway. To our knowledge, this is the first report of successful isolation and culturing of *Prorocentrum rhathymum* from this region.

[**Keywords**: *Prorocentrum,* Pigments, Arabian Sea, Chlorophyll *a*, *Dinophysis* toxin]

Introduction

Prorocentrum rhathymum is an epibenthic dinoflagellate commonly found in tropical and subtropical areas[1-5]. However, no such reports exist from the eastern Arabian Sea (Indian sector). *Prorocentrum rhathymum* has often been misinterpreted as *Prorocentrum mexicanum* in the literature due to the morphological similarity between both species. There are, however, minor differences in apical spine and trichocyst number. Some investigators[6,7] have used *P. rhathymum* as a synonym of *P. mexicanum*. *Prorocentrum rhathymum* was reinstated in 2003 as *P. rhathymum* is benthic whereas *P. mexicanum* is planktonic[2].

Although the presence of phytoplankton pigments marks the basis of photosynthetic activity (autotrophy) in phytoplankton, dinoflagellates can also have additional modes of nutrition such as mixotrophy and heterotrophy[8]. Understanding species-specific pigments give important insights into their primary mode of nutrition as well as their regional and intra/interspecies variability. They are also helpful for comparison of isolates from different geographical regions and also important for developing regional models such as CHEMTAX which is highly dependent on the input ratios of various pigments[9]. Despite earlier reports, *P. rhathymum* has not been previously isolated or

cultured from the Arabian Sea nor is its pigment composition known. Regarding toxicity, a number of investigations have reported *P. rhathymum* to be toxic. However, the true nature of the toxins was not fully resolved[10-12]. Only one report has shown production of Okadaic Acid (OA) by *P. rhathymum,* isolated from tropical waters of Malaysia[5].

Our aim in this study was to investigate the growth rates and pigment composition of *P. rhathymum* isolated for the first time from tropical waters of the Arabian Sea and establish whether it is toxic or not. To the best of our knowledge, this is the first report on successful isolation and pigment composition of *P. rhathymum* from this region.

Materials and Methods

Sea water was collected in a bucket from Dona Paula Bay area (Fig. 1) in the eastern Arabian Sea. The area has a depth of ~ 4 m; temperature and salinity during the time of collection was 27ºC and 33 PSU respectively.

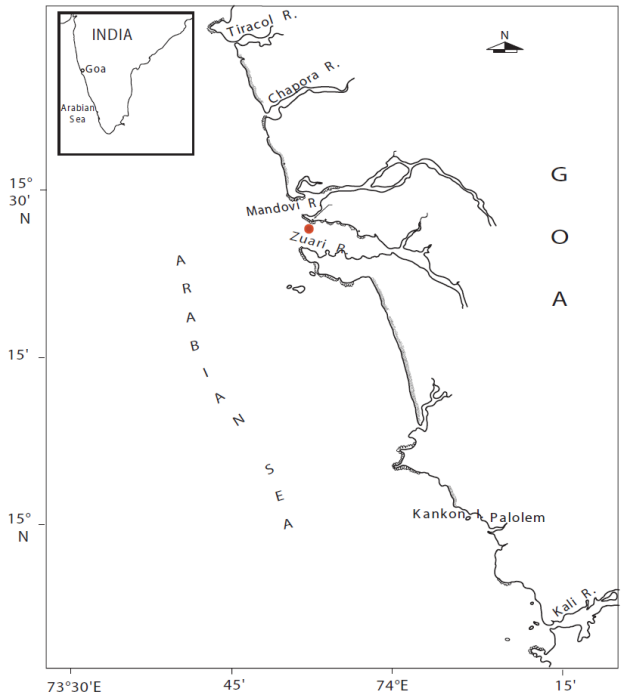

Fig.1- Map showing the sampling location in the coastal eastern Arabian Sea (red circle)

Approximately 5 liters of samples were passed through a range of sieves (100 µm, 60 µm and 10 µm) in a sequential manner and the cells collected on the 10 µm sieve were back flushed with autoclaved sea water and later observed under a microscope. A pure culture was later established by using micropipetting technique followed by repeated washing of the isolate in autoclaved seawater for proper cleanup and was designated as DPPR-1 (Dona Paula *P. rhathymum* isolate-1). During this experiment, batch cultures in triplicates were inoculated in f/2-Si[13] in Erlenmeyer flask under light level of 80 µmol photons m^{-2} $s^{-1}$with a 12h light: 12 h dark cycle by using cool-white fluorescent tubes. The culture was established from a single cell and, therefore, was clonal (strain), but not axenic. Single cultures were inoculated to give final concentrations of 20 cells ml^{-1} to begin with. For measuring the cell density, aliquots of samples (1-1.5 ml) were taken and counted using a Sedgwick-Rafter slide.

Growth rates (μ, day $^{-1}$) were calculated from microscopic cell counts over the exponential phase of growth for different cultures using the following formula[14]:

$$\mu = \frac{\ln N_1 - \ln N_0}{t_1 - t_0}$$

where N_1 and N_0 are the cell density in cells ml^{-1} at the start (t_0) and end (t_1) of the exponential phase, respectively.

Extraction of pigments was done in 3 ml of 100% acetone for 1 min using an ultrasonic probe (Labsonic U, B. Braun Biotech International, and Leverkusen, Germany) at 50 W while kept on ice to prevent excessive heating. The extracts were then stored overnight at -20°C.The entire extraction procedure was carried out in dim light and at low temperature to minimize degradation of pigments. The high performance liquid chromatography (HPLC) analysis was carried out with minor modification of calibration and preinjection procedures as mentioned below[15, 16]. A four point linear calibration was made for the standard pigments with coefficient of determination (r^2 = 0.99). A pre-injection mixture was used, where standards and samples were mixed with 28 mM solution of ammonium acetate in the ratio of 70:30(v:v); 100 µl of this mixture was injected by the autosampler. The detection limit for all the compounds varied between 0.001 to 0.002 µg l^{-1} and precision, in terms of relative standard deviation for multiple injections of standards, was well below 5 % RSD.

For Scanning Electron Microscopy (SEM), cells were post-fixed with OsO_4 for 1 h at room temperature, rinsed with distilled water, dehydrated in a graded series of ethyl alcohol concentrations followed by critical point drying. The cells were filter-mounted on a stub, air-dried and examined using a JEOL JSM 6360 LV. For light microscopy, cells were examined using an Olympus BX51 inverted microscope fitted with a digital camera. Cells were examined live, as well as after fixation with Lugol's iodine under 4X and 10X objective.

Five aliquots of cultures (approx. 50 ml each and cell concentration ~180 cells per litre) were taken during the exponential phase in Falcon tubes (Tarsons) and centrifuged for 5 min at 3200 RPM for pellet formation and all pellets were mixed together for making one sample set. This was done to get a detectable LCMS response since these toxins are known to be produced in low quantities. Supernatant was discarded and 1ml of MS grade methanol was added to the pellets and sonicated in an ice bath for 7min. The extract was re-centrifuged and the supernatant was collected for analysis after passing through 0.2 µm syringe filter as per the protocol of Agilent Technologies. LC separations were performed on Agilent 6538 UHD-Accurate-Mass Q-TOF LC-MS/MS by using Agilent HPLC-CHIP based C18 column (G4240-62006) and nano LC Agilent 1200 series with a flow rate of 0.5 $\mu lmin^{-1}$. The mobile phase was water (A) and aqueous acetonitrile (B) with 0.1 % formic acid as an additive. The LC gradient was as follows: till 2min 50% A and 50% B changed to 30% A and 70% B at the end of 4 min with a linear increase in solvent to 97% at 14min.This was mainly done to flush the chip for the next sample. The mobile phase flow rate was 0.5 $\mu lmin^{-1}$.The MS was carried out in positive full scan mode using Agilent 6538 UHD Accurate Mass Q-TOF LC-MS/MS. N_2 gas was used as drying gas for ionization at 300ºC at a flow rate of 9 $lmin^{-1}$ and compared with DTX1 standard purchased from National Research Council, Canada. The MS scan was carried in the range of 700 to 860 m/z.

Results and Discussion

Prorocentrum rhathymum was isolated from an estuarine area of the Zuari River during pre-monsoon period (March). Published biogeochemical data from the area suggests the presence of high saline water (~34 PSU) with high surface water temperature (27-33ºC) and moderate oxygen concentrations covarying with low nutrients (nitrate and phosphate)[17]**.** *Prorocentrum rhathymum*, a lesser known, potentially toxic, bloom-forming species (Fig. 2) was isolated for the first time from this region. Growth rate and pigment composition of this isolate highlight some interesting features (Figs. 3-4). The initial density of *P. rhathymum* was 20 cells ml^{-1} which increased to 238 cells ml^{-1} on day 12 and declined thereafter (Fig. 3a). Growth rate of *P. rhathymum* was 0.23 day^{-1} during the exponential phase (generally characterized by cell doubling) and decreased to 0.05 day^{-1}at the end of the experiment. This suggests that the *P. rhathymum* isolate does not reach high density, as observed in the case of *Dinophysis*[18]. Since not much information is available on growth rates of *P. rhathymum*, we compared it to *Prorocentrum lima* which has a similar ecological habitat[19,20]. Morton and Tindall[21] showed growth rates of *Prorocentrum lima* from tropical waters to range between 0.35 – 0.20 day^{-1} whereas the maximum growth rate observed for *P. rhathymum* is 0.23 day^{-1}.The cell densities observed here (Fig. 3a)are significantly lower than the reported values for *Prorocentrum* sp.[19, 21-25] which presumably may be due to the differences in genetic makeup.

The major dominant pigments found in this study were chlorophylls: chlorophyll *a*, chlorophyll c1c2, peridinin and diadinoxanthin (Fig. 3b-f). Chlorophyll *a* had a mean value of 143 µg l^{-1} and ranged between 73 – 223 µg l^{-1}(Fig. 3b). Chlorophyll *a* was 221 pg $cell^{-1}$during the initial phase and reached a maximum concentration of 237 pg $cell^{-1}$ on day 20 coinciding with the declining phase (Fig. 3c). Peridinin, a major carotenoid present in many dinoflagellates[25], was produced up to 114 pg $cell^{-1}$ (Fig. 3d); these are marginally higher than that reported (peridinin upto 40 pg $cell^{-1}$) in *P. lima*[18,25]. Chlorophyll c1c2was the next dominant marker pigment observed; it showed a similar trend as other pigments and reached a maximum concentration of 162 pg $cell^{-1}$ during the decline phase (Fig. 3e). Diadinoxanthin, which plays an important role in the xanthophylls cycle, showed a similar trend to peridinin; its values ranged between 9-29 pg $cell^{-1}$ (Fig. 3f). The diadinoxanthin marker pigment is

known to have a photoprotective role and is generally present in dark-adapted cells. It changes to diatoxanthin in high light intensities to protect the cells from photoinhibition by a process called de-epoxydation[17]. Gradual conversion of diadinoxanthin to diatoxanthin in *P. lima*cultures when grown under 70 µmol photons m^{-2} s^{-1} has been observed[19]. However, we did not observe any buildup of diatoxanthin in our samples grown under light levels of 80 µmol photons m^{-2} s^{-1}. This shows that such physiological changes may also be species-specific. Mean ratio of Chl c1c2: Chl *a* was found to be 0.56and ranged between 0.40-0.70 (Fig. 4a). The mean ratio for Peridinin: Chl *a* was 0.28, ranging between 0.24-0.4 (Fig. 4b). Diadinoxanthin: Chlorophyll *a* ratio was low and had a mean value of 0.07 (Fig.4c). However, all the ratios showed an increasing trend in tandem with the growth curve (Fig. 3a). Beta-carotene was not detected in our samples. Similarly, pigments such as diatoxanthin or 19'-butanoyloxyfucoxanthin reported to be present in dinoflagellates such as *Gymnodinium* sp. and *Amphidinium*sp.[27,28] were also below detectable levels. The peridinin to chlorophyll *a* ratio is similar to an earlier observation[21]. This suggests that although the magnitude of these pigments produced varies between isolates, their relative proportions with respect to chlorophyll *a* may be constant.

Qualitative analysis by LC-MS/MS in positive ESI mode confirmed presence of DTX1 which was further compared with standard having precursor ion of m/z 841.56 (Fig. 5). This suggests that *P. rhathymum*, isolated from this region, is toxic and presumably genetically different than the isolate from coastal waters of Malaysia[5] which produces OA in higher amounts than DTX. Since the number of cells isolated was less, it may be likely that the other toxins such as OA or DTX2 were not detected and should be further corroborated with specific experiments.

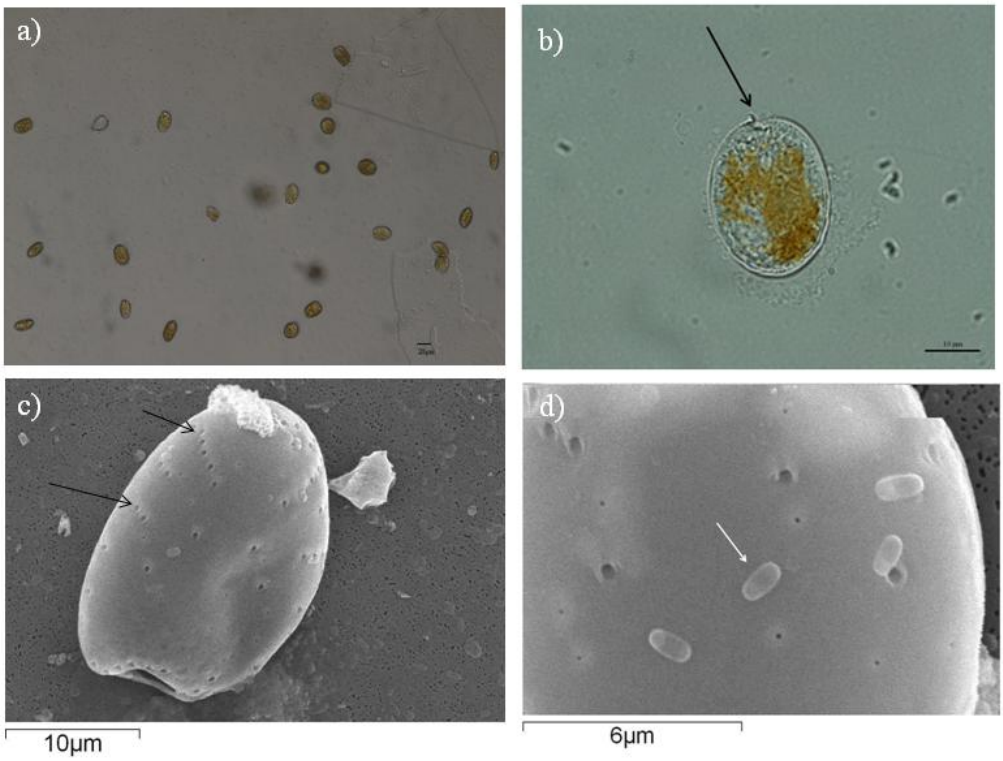

Fig.2- *Prorocentrum rhathymum*. a) Light microscopic (LM) view of the cells growing in the tissue culture plate; b) LM view of the periflagellar collar curved and prominent (arrow head) scale 10µm; c) SEM view of the trichocyst pores radially arranged (arrowheads); d) SEM view of the cell surface showing associated bacteria (arrowhead).

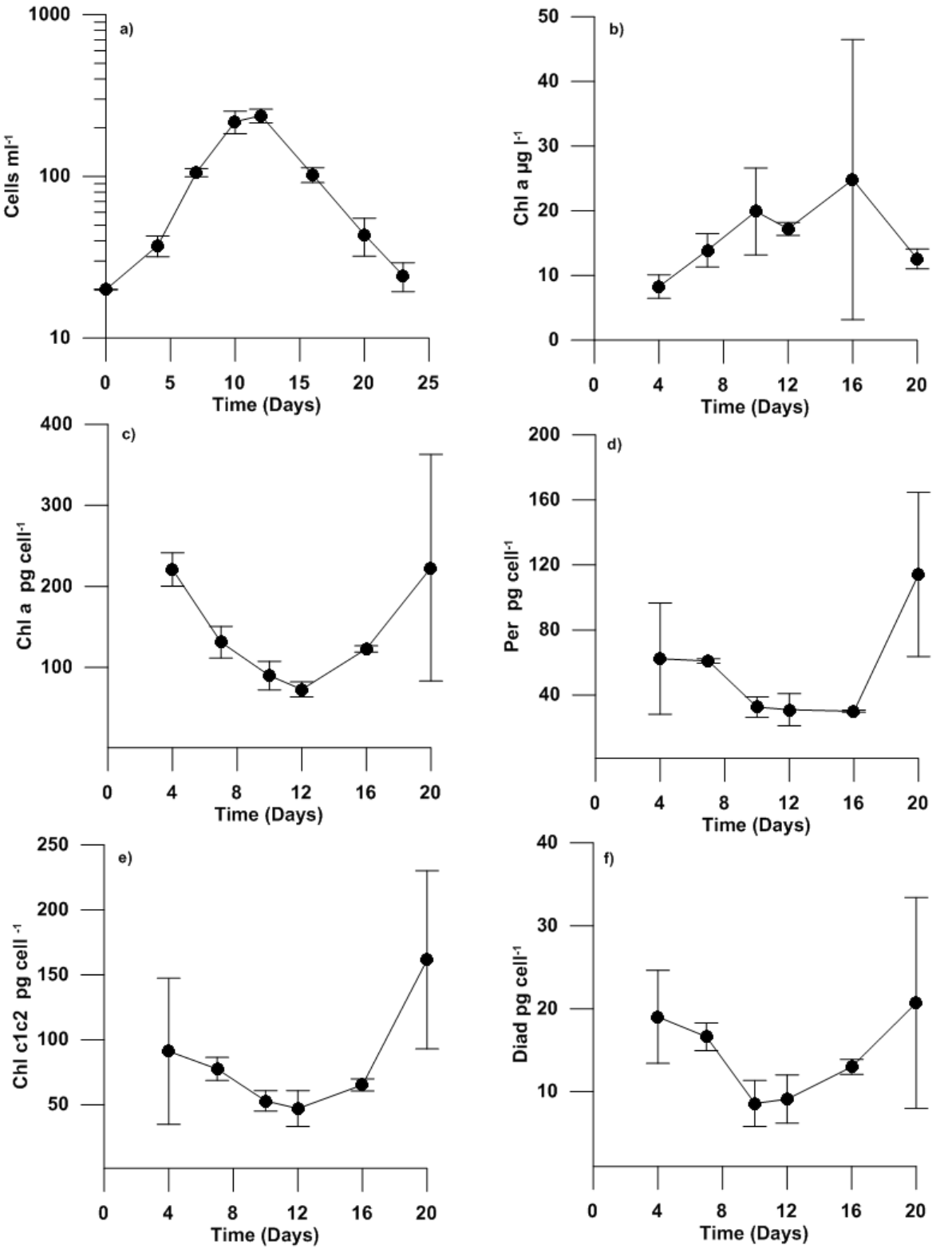

Fig.3-Profiles showing temporal variations in the abundance and major phytoplankton pigments observed in *P. rhathymum* (in log scale).a) cell abundance (cells ml^{-1}); b) Chl *a* (µg l^{-1}); c) Chl *a* (pg $cell^{-1}$); d) Peridinin (pg $cell^{-1}$); e) Chl c1c2 (pg $cell^{-1}$); f) Diadinoxanthin (pg $cell^{-1}$). Error bars drawn from triplicate measurements (n=3).

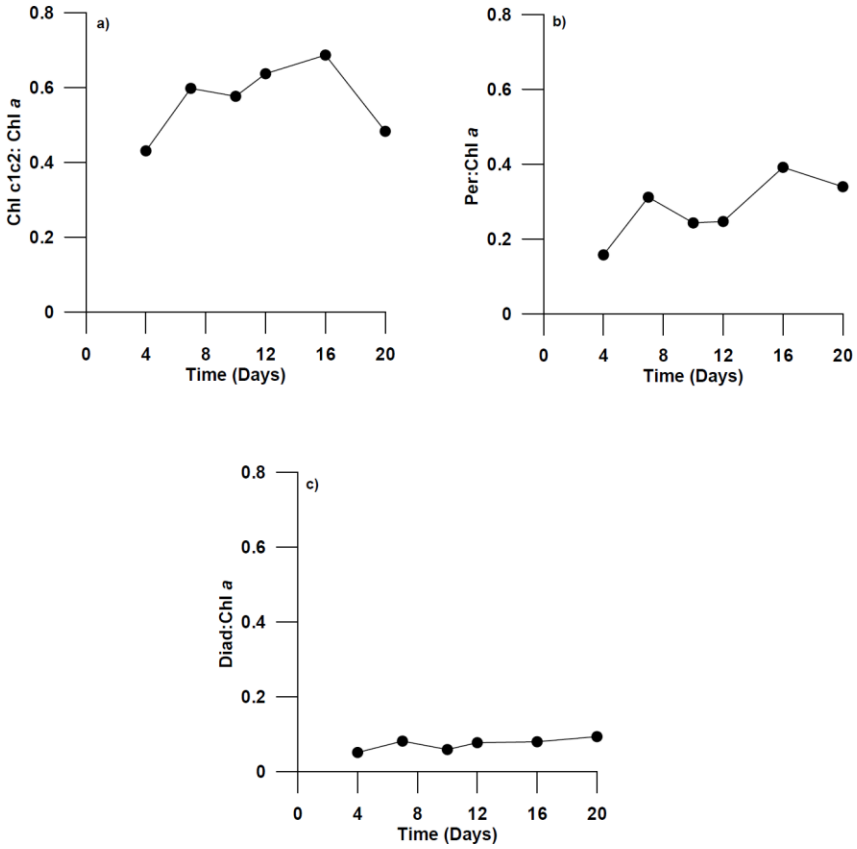

Fig.4-Profiles showing temporal variability in the pigment ratios observed in *P. rhathymum.* a) Chl c1c2: Chl *a*; b) Peridinin: Chl *a;* c) Diadinoxanthin: Chl *a*.

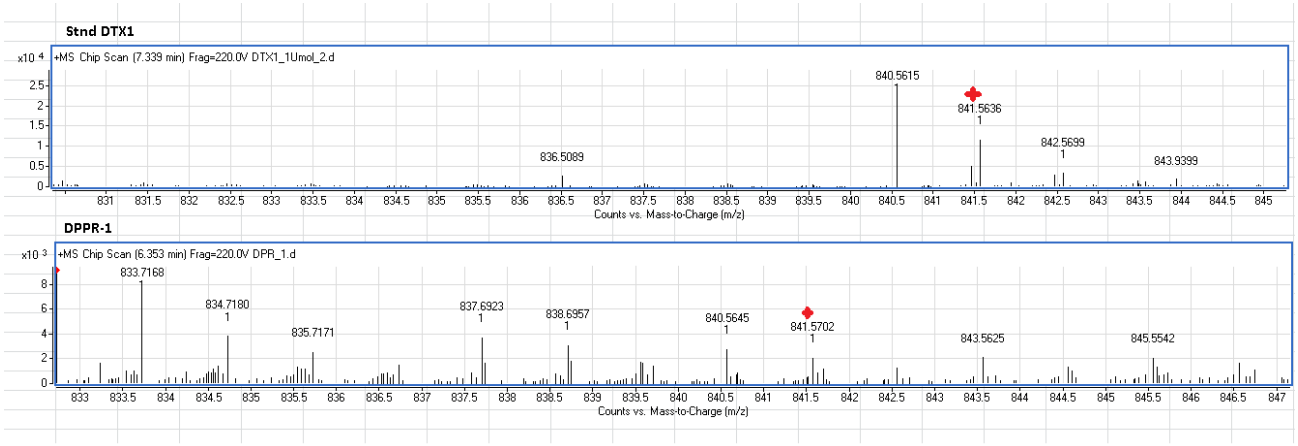

Fig.5-LC-MS/MS Spectrum of a) DTX1 standard (m/z 841.56) b) extract from the isolate *P. rhathymum* run in positive ESI mode in Agilent 6538 UHD-Accurate-Mass Q-TOF. Red star indicates the precursor ion.

In conclusion, clonal strains of *P. rhathymum* were isolated for the first time from the coastal eastern Arabian Sea. LC-MS/MS data showed production of DTX1. This suggests the isolate is toxic in nature and therefore should be further investigated. Phytoplankton pigment studies revealed presence of chlorophyll *a*, chlorophyllc1c2, peridinin and diadinoxanthin. Peridinin to chlorophyll *a* ratio was found to be similar to the reported values from other *Prorocentrum* sp. Further studies to identify its molecular diversity along with toxicity are presently underway.

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