Changes in intracellular and extracellular free polyamines during the growth cycle of *Prorocentrum donghaiense*

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Abstract. We used HPLC to measure the concentrations of free putrescine, spermidine, and spermine in cells and culture medium during the growth of *Prorocentrum donghaiense*. Spermine was the main intracellular free polyamines in *Prorocentrum donghaiense*. The intracellular concentration of free polyamines kept increasing during the growth cycle and the ratio of spermine to putrescine increased with cell density. Changes in the ratios of spermine/spermidine in *Prorocentrum donghaiense*, however, occurred before changes in cell density, suggesting that polyamines can regulate cell growth and replication. Putrescine was the most abundant free polyamine in the culture medium, followed by spermine and spermidine. Changes to the media polyamines contents implied that *Prorocentrum donghaiense* could absorb free polyamines from the culture solution as well as release free polyamines into the medium during different growth periods, especially during the decline phase. These results suggest that masses of dead algae in the population decline phase would release abundant free polyamines into the environment.

1. Introduction
The Polyamines putrescine (Put), spermidine (Spd), and spermine (Spm) are low molecular weight, aliphatic polycations found in cells of all living organisms [1]. They are essential for regulating cell growth, proliferation, gene expression, and translation [2,3].

There have been some reports suggesting a relationship between exogenous polyamines and algae growth. Putrescine at concentrations ranging from 0.1 to 5.0 μM stimulated the growth of *Gymnodinium mikimotoi* [4]. The growth of *Microcystis aeruginosa* in putrescine or spermine at 25 μM ranged from 145% to 175% above that observed in 25 μM NO₃⁻ [5]. Putrescine concentrations from 1.0 to 110 μM in culture medium enhanced the growth of *Chrysochromulina lesdbeateri*; however concentrations higher than 110 μM inhibited growth [6].

Changes in intracellular polyamines concentrations have also been reported in algae during the
growth cycle. The free form of spermidine was the most abundant polyamine in Heterosigma akashiwo cells during the growth period except for the lag phase [7]. Putrescine in the conjugated form increased during the exponential growth phase of Alexandrium minutum. A 10-20 fold increase in free putrescine and spermidine was observed in Chlamydomonas reinhardtii and Scenedesmus obliquus during the growth cycle [8,9]. In contrast, polyamines appear to enhance the toxicity of ichthyotoxin in prymnesium parvum and the haemolytic activity of Chrysochromulina lesbeateri.

Early in 1984, polyamines were shown to stimulate and regulate the growth of bloom-forming phytoplankton [10]. Subsequently, a possible link between the release of putrescine from the decay of the spring diatom bloom and blooming of Gymnodinium nagasakiense was reported. Limited experimental reports suggested that polyamines are an important factor regulating the red tide. In recent years, the East China Sea has become an area of high red tide occurrence [11], causing extensive damage to the environment. Prorocentrum donghaiense is one of the dominant species in the East China Sea and can reach high densities in spring, summer, and autumn under appropriate conditions [12]. In this study, the concentrations of free putrescine, spermidine, and spermine in Prorocentrum donghaiense cells and in the culture media were monitored over the life cycle to explore changes in polyamines levels and possible importance for its development during the growth cycle.

2. Materials and methods

2.1. Algae culture

Prorocentrum donghaiense was supplied from the algae collection of the Institute of Oceanology, Chinese Academy of Science. Cells were cultured in f/2 medium with 1L erlenmeyer flask [13] at 20 ± 1°C under a 12 h light: 12 h dark cycle with a light intensity of 3000 lux in illumination incubator (labCAN, LPGZ-250D). The initial inoculation density was 10^7 cell/L. A portion of the microalgae suspension was withdrawn every 2-3 days and cells were counted by microscope (OLYMPUS, CX23).

2.2. The determination of polyamines in microalgae cells

The cells in 4 mL of the suspension were harvested every 2-3 days between 9:00 and 10:30 by centrifugation at 2000xg for 20 minutes withcentrifugal machine (Pingfan, GL-26MC). The cell pellet was sonicated in 1 mL 5% perchloric acid (PCA, 70%, SINOPHARM) for 5 minutes and then recentrifuged for 5 minutes. The supernatant was used for free polyamine determination. To label polyamines for fluorescence detection, 1 mL of supernatant with 20 µL 10^-6 M 1,6-hexylenediamime (DAH, AR, SINOPHARM) as an internal standard was mixed with 2 mL saturated sodium carbonate (Na₂CO₃, AR, SINOPHARM) and 2 mL dansyl-chloride(>=99%, Sigma) (6 mg/mL in acetone(C₃H₆O,GR, Merck)). After vortexing the mixtures for 30 seconds, the mixture were incubated in the dark for 45 minutes at 40°C. The reaction was stopped by the addition of 100 µL of 25% ammonia(NH₃·H₂O, AR, SINOPHARM) in water followed by incubation in the dark for 30 minutes. Finally, the dansylated polyamine was extracted with diethyl ether(C₄H₁₀O, AR, SINOPHARM) and the ether layers were evaporated to dryness under a steam of nitrogen. The residues were dissolved in 300 µL acetonitrile (C₂H₅N, GR, Merck) and analyzed by HPLC.

2.3. The determination of polyamines in culture medium

The culture medium was filtered through glass microfibres (GF/F, Whatman, φ 25 mm) and pre-combusted at 450°C for 4.5 hours. Then, 1 mL of filtrate with 20 µL 10-6 M 1,6-hexylenediamime (DAH) as an internal standard was mixed with 15 µL 70% PCA, incubated at 4°C for 30 minutes, and then mixed with 45 µL 2 M sodium hydroxide and 70 µL borate buffer (pH 9.18, ≥98%, SINOPHARM). After vortexing this mixture for 30 seconds, it was incubated in the dark for 45 minutes at 40°C. The reaction was stopped by the addition of 100 µL 25% ammonia(NH₃·H₂O, AR, SINOPHARM) in water followed by incubation in the dark for 30 minutes. Finally, 60 µL of acetonitrile was added and the mixture analyzed by HPLC.
2.4. Chromatographic conditions

For chromatographic detection, a Waters e2695 high-performance liquid chromatograph was used with a C18 column (150 mm × 4.6 mm i.d., 5 µm particle size, Kromasil, Sweden) and a Waters e2475 Fluorescence detector (Waters, Milford, USA). The fluorescence of the polyamine conjugates was measured at 340 nm for the excitation wavelength and 515 nm for the emission wavelength. The mobile phase solution A was 0.1 mol/L NH₄Ac (AR, SINOPHARM) and mobile phase solution B was acetonitrile. Prior to use, the mobile phase solutions were filtrated through 0.45 µm GF/F and pre-combusted at 450º C for 4.5 hours. Gradient elution started at 35% A and 65% B and changed as follows: 0-10 min, 35% A to 60% A; 10-15 min, 60% A to 80% A; 15-20 min, 80% A to 100% A; 20 to 30 min, 100% A to 35% A (table 1). The temperature of the column was kept at 40° C and the flow rate was 1.0 mL/min. The injection volume was 10 µL for all samples.

| elution time(min) | A(%) | B(%) |
|------------------|------|------|
| 0                | 35   | 65   |
| 10               | 60   | 40   |
| 15               | 80   | 20   |
| 20               | 100  | 0    |
| 30               | 35   | 65   |

3. Results

3.1. Variation of free polyamine in the prorocentrum donghaiense cell

Samples were taken for eight times, two parallel samples each time, 18 samples in total. Free spermine was the most abundant intracellular polyamine in Prorocentrum donghaiense, followed by putrescine and spermidine (figure 1). The content of free spermine increased from 21 fmol/cell on the fourth day to 70 fmol/cell on the seventeenth day, while putrescine rose from 3 fmol/cell to 19 fmol/cell from day 3 to day 17. In contrast, spermidine did not change as significantly, from 2 fmol/cell to 7 fmol/cell.

Spermine and putrescine were the main free polyamines in Prorocentrum donghaiense cells. The concentrations of all three free polyamines increased over the entire growth period, especially during the decline phase (figure 1), when concentrations rose dramatically. This continuous increase in
Prorocentrum donghaiense polyamine content is significantly different to the changes observed in Heterosigma akashiwo and Alexandrium minutum. The most abundant free polyamine in Heterosigma akashiwo was spermidine, followed by putrescine and spermine, and the contents of all three species increased only transiently during the initial growth phase [7]. The most abundant free polyamine in Alexandrium minutum was putrescine followed by spermine and spermidine, and the content of these polyamines decreased quickly during the growth cycle with only a slightly increase during the decline phase [12].

3.2. Variation of free polyamine in culture medium
The most abundant free polyamines in the culture medium were putrescine and spermine. The content of putrescine decreased during the early culture stage from an initial concentration of 114 nM to 90 nM by the fifth day. Putrescine showed a more dramatic increase during the declining phase that was preceded by a slight invariably increase. The content of free spermine in the culture medium decreased continuously except for a modest increase during the decline phase. The content of spermidine was the lowest of the three polyamines over the entire growth cycle and did not change significantly (figure 2).

4. Discussion
As small cationic molecules, polyamines regulate the biosynthesis proteins, RNA, and DNA, and play a role in cell proliferation and the modulation of cellular signalling [14,15]. The contents of three polyamines in Prorocentrum donghaiense cells increased moderately during the positive phase of growth cycle, and then increased dramatically during the decline phase. In the exponential growth phase, the increase in intracellular free polyamines correlates with active DNA biosynthesis. A higher rate of increase during the decline phase might reflect a protective mechanism against various stressors. Indeed, there have been many reports demonstrating that polyamines accumulate in response to abiotic and biotic stresses [16-18], such as pathogen invasion, oxidation, and acidification [19,20]. In addition, plants over-expressing polyamine biosynthetic genes showed improved stress tolerance [21-23]. During the decline phase, polyamine biosynthetic genes that are sensitive to nutrient stress might also be over-expressed to enhance survival.

Spermine and putrescine were the main free polyamines in Prorocentrum donghaiense, while the content of spermidine was far lower. Spermine and putrescine can stimulate cell division and differentiation [4,10], and the ratio of Put/Spd synchronously changed over the cell cycle [24]. In our experiments, the ratio of Spm/Spd in Prorocentrum donghaiense (figure 3) was positively correlated with cell density ($R^2=0.9072$), again suggesting that putrescine synthesis rises in synchrony with DNA
synthesis. Changes in the ratio of free Spm/Spd in Prorocentrum donghaiense (figure 4) preceded changes in cell density, underscoring the possibility that these polyamines can regulate cell growth.

Putrescine and Spermine were also the main free polyamines in the culture medium. The sharp decreases in extracellular free putrescine and spermidine during the early culture stage indicates that Prorocentrum donghaiense can absorb free polyamines from the culture medium. In contrast, the increasing extracellular putrescine during the exponential growth phase implies that Prorocentrum donghaiense can release free polyamine into culture medium when metabolism is accelerated. A slightly lower content of free putrescine in the early stage of the decline phase might be due to metabolic slowdown. The sharp increase in extracellular free putrescine during the decline phase could reflect the release of free putrescine into the environment from dead algae. Similarly, dying algae would also release spermidine and spermine. The decrease of three polyamines in the late culture stage might due to polyamine degradation and uptake by bacteria in waters.

5. Conclusions
Spermine and putrescine and were the main intracellular free polyamines in Prorocentrum donghaiense. The concentrations of free polyamines increased continuously over the entire growth cycle. The dramatically higher increase in free intracellular polyamines during the decline phase might due to the over-expression polyamine biosynthetic genes under stress. The ratio of spermine to putrescine in Prorocentrum donghaiense was positively correlated to changes in cell density, which in turn reflects changes in the cell cycle. However, changes in the spermine/spermidine ratio preceded changes in cell density, consistent with the idea that polyamines can regulate cell growth. Sea seawater during significant 2010 algal blooms were spermine, putrescine and spermidine, it implied that the Prorocentrum donghaiense blooms had a closed relationship with polyamines.

Putrescine was the most abundant free polyamine in the culture solution, followed by spermine and spermidine. Changes in extracellular polyamines implied that Prorocentrum donghaiense could absorb free polyamines from the culture solution as well as release free polyamines into the extracellular environment during specific phases of the growth period. The mass death of algae during the decline phase released large amounts of free polyamine into the environment.

Acknowledgment
We are thankful to the National Natural Science Foundation of China and National 973 project of China for their financial support. The authors appreciate the help of Prof. Wang Jiangtao.

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