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# Risk assessment of nitrate and oxytetracycline addition on coastal ecosystem functions

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ABSTRACT

Diatoms dominate phytoplankton communities in the well-mixed coastal and upwelling regions. Coastal diatoms are often exposed to both aquaculture pollution and eutrophication. But how these exposures influence on coastal ecosystem functions are unknown. To examine these influences, a coastal centric diatom, *Conticribra weissflogii* was maintained at different concentrations of nitrate (N) and/or oxytetracycline (OTC). Algal density, cell growth cycle, protein, chlorophyll *a*, superoxide dismutase (SOD) activity, and malonaldehyde (MDA) were determined for the assessment of algal biomass, lifetime, nutritional value, photosynthesis and respiration, antioxidant capacity, and lipid peroxidation, respectively. When N addition was combined with OTC pollution, the cell growth cycles were shortened by 56–73%; algal density, SOD activities, the concentrations of chlorophyll *a*, protein, and MDA varied between 73 and 121%, 19 and 397%, 52 and 693%, 19 and 875%, and 66 and 2733% of the values observed in N addition experiments, respectively. According to *P*-value analysis, the influence of OTC on algal density and SOD activity was not significant, but the effect on cell growth cycle, protein, chlorophyll *a*, and MDA were significant ($P < 0.05$). The influence of N addition with simultaneous OTC pollution on the above six end points was significant. Algal biomass, lifetime, nutrition, antioxidant capacity, lipid peroxidation, photosynthesis, and respiration were all affected by the addition of OTC and N. Coastal ecosystem functions were severely affected by N and OTC additions, and the influence was increased in the order: N < OTC < OTC + N. Thus, the simultaneous monitoring of N and OTC was necessary for the risk assessment of aquaculture pollution on coastal ecosystem functions.



## 1. Introduction

Coastal ecosystems play an important role in global biogeochemical cycles and may contribute between 36% and 77% of global ecosystem services value (Costanza, 1999; Hegedüs et al., 2001; Martínez et al., 2007; Liquete et al., 2013). Diatoms are the dominant group of phytoplankton in the modern ocean, especially in well-mixed coastal and upwelling regions (Armbrust, 2009). They account for approximately 40% of oceanic primary productivity and over 50% of organic carbon burial in marine sediments (Falkowski et al., 2004; Rabosky and Sorhannus, 2009). The coexistence of aquaculture pollution and eutrophication has been reported in coastal environments (Miranda and Zemelmen, 2002; Rigos et al., 2002, 2003; Porrello et al., 2003a,b; Delépée et al., 2004; Reed et al., 2004; Ueno et al., 2004; Zuo et al., 2006; Fritz and Zuo, 2007; Herbeck et al., 2012). How coastal diatoms will respond to the rapidly changing coastal conditions is critical for the health of coastal environments.

Macronutrient enrichment can influence algal growth, cell shape, dry weight per cell, basic functional groups in marine algae, and the contents of carbohydrate, protein, and chlorophyll *a* (Kucuksezgin et al., 2006; Li et al., 2007; Point et al., 2011). The growth of planktonic algae (*Chlorella vulgaris* and *Microcystis aeruginosa*) could be affected by aquaculture pollution (e.g., oxytetracycline (OTC), streptomycin) (Zhao et al., 2005; Carney et al., 2011; Qian et al., 2010). However, the influence of the coexistence of aquaculture pollution and eutrophication on coastal ecosystem functions is unknown. At the same time, how these exposures affect the formation of free radicals from coastal diatoms and the subsequent induction of a protective mechanism have not been studied thoroughly, either.

Algal density, cell growth cycles, protein, chlorophyll *a*, superoxide dismutase (SOD) activity, and malonaldehyde (MDA) content

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have been used in our laboratory for the estimation of algal biomass, lifetime, nutritional value, photosynthesis and respiration, antioxidant capacity, and lipid peroxidation, respectively, because: (a) algal density and growth cycles control the stability of food chains and the energy transfer in the food chains of marine systems; (b) protein synthesis is one of nitrogen assimilations and its concentration is an indicator of metabolic capacity and nutritional value; (c) chlorophyll *a* is an important material for algal photosynthesis and respiration, and its concentration is an important indicator for estimating algal primary productivity (Li et al., 2006); (d) SOD is one kind of metalloenzyme that can catalyze the dismutation process of superoxide anion into oxygen and hydrogen peroxide, Thus, SOD provides protection for the survival of aerobic organisms (Choudhary et al., 2007; Martínez et al., 2007; Learman et al., 2011); (e) MDA, a product of lipid peroxidation, can be used as an indicator of free radical activity and tissue damage (Jones, 1984). These six end-points were used for the risk assessment of the coexistence of aquaculture pollution and eutrophication on coastal ecosystem functions in this study.

A coastal centric diatom, *Conticribra weissflogii*, has been used as a model of diatoms (Qu et al., 2000) and a good indicator species for toxicity assessment of marine contaminants (Onwurah et al., 2007). Macronutrients in the coastal and shelf waters include nitrogen (nitrate, nitrite, ammonium, dissolved organic nitrogen) and phosphorus (phosphate, and dissolved organic phosphorus), while nitrate (N) and phosphate (P) are the predominant species (Dafner et al., 2007). Oxytetracycline (OTC) is a broad-spectrum antibacterial agent that belongs to the tetracycline antibacterial group. It has been used all over the world for decades for the treatment of bacterial diseases in aquaculture (Rigos et al., 2004; Reiser et al., 2011). OTC has been used as a model of aquaculture pollutants in marine ecosystem (Samuelsen, 1989; Elema et al., 1996; Zhao et al., 2010). Therefore in this study, we used *C. weissflogii* as a model of coastal diatoms, nitrate as nitrogen, phosphate as phosphorus, and OTC as aquaculture pollutant.

## 2. Materials and methods

### 2.1. Instrumentation

UV-3200PCS UV–Vis spectrophotometer (Shanghai Spectrum Instruments Co., China), double-sided clean bench (Suzhou Purification Equipment Co., China), SPX-300 IC Microcomputer artificial climate chamber (Shanghai Bo Xun Industrial Co., China), Branson-102C ultrasonic crushing device (Branson Ultrasonic Co., China), Leica DM LB2 microscope Leica (Leica Instruments, Germany), MK-III-based fiber optic pressure controlled closed microwave digestion system (Shanghai Branch Microwave Digestion Test New Technology Institute, China), and Milli-Q ultrapure water system (Millipore company, USA) were used in this study.

### 2.2. Chemicals and standard solutions

All reagent solutions were made in water (>18 MW $cm^{-1}$) purified by a Milli-Q (MQ) ultrapure water system and stored at 4 °C. $NaNO_3$, $Na_2HPO_4$, EDTA-$Na_2$, $NH_4Cl$, OTC, ammonium molybdate, ascorbic acid, sulfanilic acid, and *N*-(1-naphthyl) ethylenediamine dihydrochloride were analytical grade (Sigma, USA). Nitrate and phosphate standard solutions were prepared from stock standard solutions of $NaNO_3$ (N, 10 mM) and $Na_2HPO_4$ (P, 360 mM), respectively. The stock solution of 1000 mg $L^{-1}$ OTC was prepared in water.

### 2.3. Seawater and marine phytoplankton cultures

Seawater was collected from the Eastern China Sea, stored at 4 °C for about 6 months, and filtered through acid-washed Pall Acropak Supor capsule 0.22 μm filters before use. The N and P concentrations in the seawater were determined in triplicates using a flow injection analyzer (FIA), and the concentration of N (as nitrate) and P (as reactive P) was 1.2 and 0.2 μmol $L^{-1}$, respectively.

In coastal environment, *n* (N):*n* (P) was in the range of 8:1 to 64:1 (Li et al., 2006, 2008; Wang et al., 2007). Unialgal cultures of *C. weissflogii* were obtained from the State Key Laboratory for Marine Environmental Science, Xiamen University. Under the light illumination of 140 μmol photons $m^{-2}$ $s^{-1}$ by a light:dark cycle as 14–10 h, they were maintained in seawater containing 21.1 mmol $L^{-1}$ Si (by adding $Na_2SiO_3 \cdot H_2O$), 1.0 μmol $L^{-1}$ P, various concentrations of N (8.0, 16.0, 32.0, and 64.0 μmol $L^{-1}$, respectively) and/or OTC (0, 1.0, 3.0, 5.0, 10.0 mg $L^{-1}$, respectively) at 19 °C. After determination of N, P, and OTC in the medium, their concentrations were maintained through compensating addition daily of $NaNO_3$, $Na_2HPO_4$, and OTC for one life cycle, i.e., semi-continuous culture was adopted. The algal suspensions were stirred at 100 rpm to simulate the current of seawater and to reduce the adsorption of OTC by vessel. These experiments were triplicates ($n = 3$).

### 2.4. Test methods

Algal density was counted microscopically. When algal density reached its maximum, algae were collected onto a 0.2 μm membrane filter. These algae were extracted by acetone (5.0 mL, 90% v/v) for 10 h under freezing and dark conditions and then centrifuged for 10 min at 7000 rpm. The absorbance of the supernatant was measured at four separate wavelengths, 663, 645, 630, and 750 nm, chlorophyll *a* concentrations were calculated using the equations described by Royer et al. (2008), and its detection limit was 1.6 μg $L^{-1}$.

Algal cells were added into 5.0 mL of cold phosphate buffer (0.1 mol $L^{-1}$, pH = 7.8) in an ice bath, ruptured fully by ultrasonic fragmentation, and then the homogenate was centrifuged at 15,000 rpm at 4 °C. The supernatant was used for enzyme assay and the determination of protein and MDA. Protein was determined by coomassie brilliant blue method (Li et al., 2010). SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium chloride (Choudhary et al., 2007). MDA content was estimated using thiobarbituric acid method (Csiszár et al., 2007). The detection limit of protein, SOD activity, and MDA was 1.0 μg $L^{-1}$, 0.1 U (cell FW h)$^{-1}$, and 1.0 μg $L^{-1}$, respectively.

### 2.5. Statistical approaches

Analysis of variance was calculated by using SASPROC MIXED (Littell et al., 1996) and *P* value was calculated using two-way ANOVA. For all analyses, significance was assigned at the $P < 0.05$ level.

## 3. Results

### 3.1. Influence of N and OTC addition on algal density

Cell growth rate was controlled by the speed of cell division and directly affected by N concentration. The results shown in Fig. 1 indicated that N addition in the range of 8.0 to 64.0 μmol $L^{-1}$ could stimulate cell growth and such stimulating effect was the most significant at 32.0 μmol $L^{-1}$ N. OTC addition with different