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# A community-based approach to identifying defence of microalgae against protozoan grazing

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*It has increasingly been recognized that defence of microalgae against predator grazing is a passive response to increase algal population density by excreting chemicals with a change in physical properties. As common biological pollutants in the cultivation of the microalgae, the community-based method was used to identify the ability of two microalgae,* Chlorella *sp. and* Nannochloropsis oceanica, *to defend against protozoan grazing. Mature protozoan samples with 14-day age were collected, using microscopy glass slides, in coastal waters of the Yellow Sea, northern China. For both microalgae, a gradient of concentrations was designed as $10^0$ (control), $10^4$, $10^5$, $10^6$ and $10^7$ cell $ml^{-1}$, respectively. Results showed that both test algal species represented strong defence effects on protozoan grazing, especially at high density levels. Species richness, abundance and taxonomic distinctness of the protozoan assemblages showed a sharp decrease at high concentration level ($10^7$ cell $ml^{-1}$) of both algae. A significant variation in protozoan community structures was found to be driven by the gradient of the algal concentrations. The paired taxonomic distinctness indices of the protozoan communities showed an increasing trend of departure from the expected taxonomic pattern with increase of algal concentrations. Based on the results, we suggest that the community-based bioassay might be used as a feasible tool for identifying defence against protozoan grazing of microalgae.*

**Keywords:** bioassay, protozoan community, biodiversity, taxonomic distinctness, *Nannochloropsis oceanica*, *Chlorella* sp.

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## INTRODUCTION

Microalgal mass culture technologies have failed to produce bulk volumes of microalgal biomass at low cost due to contamination by biological pollutants (Zou & Richmond, 1999; Hu & Gao, 2003; Aslan & Kapdan, 2006; Chisti, 2007; Park *et al.*, 2011; Day *et al.*, 2012; Peng *et al.*, 2015). Of these biological pollutants, protozoa are the common predatory species in the mass cultivation of microalgae (Rosetta & McManus, 2003; Frederiksen *et al.*, 2006; Li *et al.*, 2006; Lurling & Beekman, 2006).

Recently, it has been recognized that many microalgae have a defence against predator grazing (Wang *et al.*, 2013). Previous investigations have demonstrated that the defence is a passive response to increased algal population density by excretion of chemicals with a change in physical properties (Wang *et al.*, 2013). However, as regards multivariate approaches to identifying their ability of defence little information has been documented.

As a primary component of microbiota, protozoa play a crucial role in transferring carbon and energy from low tropic levels (bacteria and microalgae) to high tropic levels in microbial food webs (Norf *et al.*, 2009a, b; Xu *et al.*, 2014). With short generation times, relative immobility, rapid responses to environmental changes and ease of sampling, they have been widely used as a robust bioindicator for bioassessment or bioassay, especially at community level (Xu *et al.*, 2014).

In this study, the defence effects of microalgae on protozoan grazing were studied at community level using an artificial substratum method. The main objectives of this study were: (1) to reveal the defence effect of microalgae on protozoan grazing, (2) to demonstrate the relationships between variations in species richness, taxonomic distinctness of protozoan communities and algal concentrations, and (3) to evaluate the feasibility of community-based bioassay for identifying the defence against protozoan grazing.

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## MATERIALS AND METHODS

### Protozoan sample collection

Samples of protozoan communities were collected in coastal waters of the Yellow Sea, northern China (Figure 1). The glass slide systems were designed, deployed, anchored and sampled as described by Xu *et al.* (2011b). A total of 20 microscopy glass slides were used as artificial substrata for collecting the protozoa at a depth of 1 m below the water surface. Two PVC frames were used to hold the 20 glass slides and all slides were collected at the exposure time of 14 days. The glass



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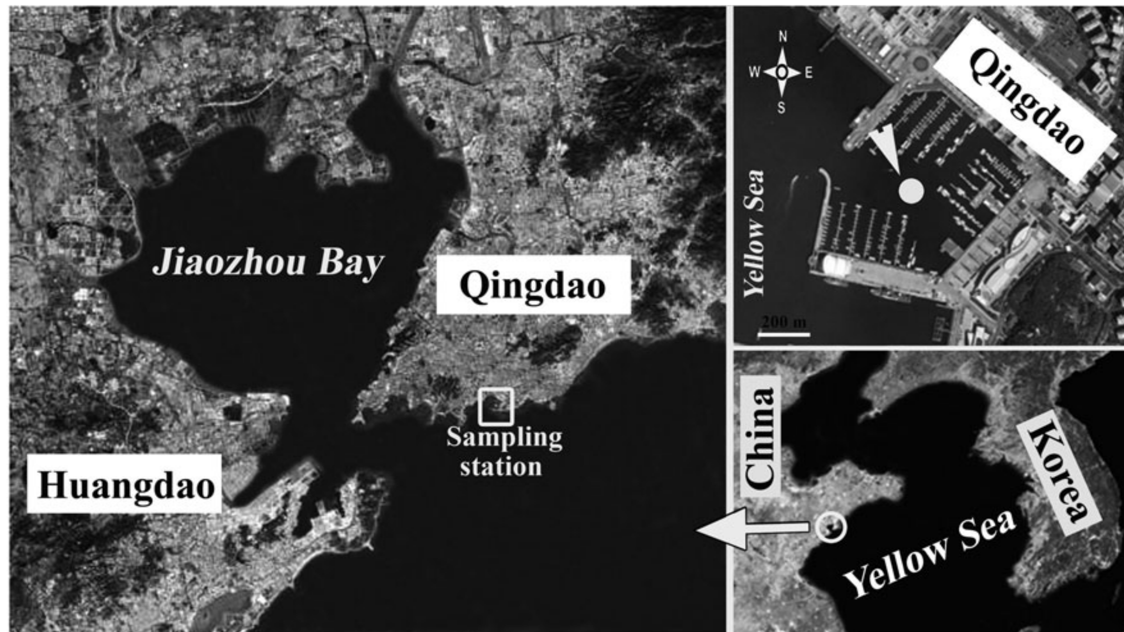


**Fig. 1.** Sampling station, which was located in the harbour of the Olympic Sailing Center (OSC), a coastal area of the Yellow Sea, near Qingdao, northern China.

slides were transferred into Petri dishes containing *in situ* water and then stored in a cooling box before transporting to the laboratory within 2 h for testing of protozoan assemblages (Xu *et al*., 2014).

After 3-day domestication under laboratory conditions in an illumination culture cabinet (temperature 21.6°C and illumination 3960 LUX), a total of 18 glass slides with protozoan communities were used as test communities.

**Table 1.** Protozoan (mainly ciliate) species identified in samples used and average abundance (ind. $cm^{-2}$) in five (1, 2, 3, 4 and 5) treatments with five ($10^0$, $10^4$, $10^5$, $10^6$ and $10^7$ cell $ml^{-1}$) concentrations of two algal species *Nannochloropsis oceanica* and *Chlorella vulgaris*, respectively. Protozoan abundance: + = 0–1, ++ = 1–10, +++ = 10–100, ++++ = 100–1000, +++++: over 1000 ind $cm^{-2}$). C = *Chlorella vulgaris*; N = *Nannochloropsis oceanica*.

| Species | 1 | 2 (C) | 3 (C) | 4 (C) | 5 (C) | 2 (N) | 3 (N) | 4 (N) | 5 (N) |
|---|---|---|---|---|---|---|---|---|---|
| *Acineria incurvata* | + | + | + | − | − | + | + | + | − |
| *Anteholosticha warreni* | + | − | − | − | − | − | ++ | + | − |
| *Apoamphileptus robertsi* | − | + | − | − | − | − | + | − | − |
| *Aspidisca aculeata* | + | + | + | + | − | ++ | + | + | − |
| *Aspidisca magna* | + | − | + | − | − | − | + | − | − |
| *Aspidisca steini* | − | − | + | − | − | + | + | + | − |
| *Coeloperix sleighi* | + | + | + | + | − | + | + | + | − |
| *Diophrys appendiculata* | ++++ | ++++ | − | +++ | ++ | ++++ | ++ | +++ | + |
| *Dysteria pectinata* | ++ | ++ | ++ | + | − | ++ | + | ++ | ++ |
| *Dysteria semilunaris* | + | + | − | + | − | + | − | + | − |
| *Folliculina simplex* | + | + | − | + | − | + | + | + | + |
| *Hartmannula angustipilosa* | − | + | − | + | − | − | + | + | − |
| *Hartmannula derouxi* | − | + | + | + | − | + | + | − | − |
| *Hemigastrostyla enigmatica* | + | + | − | + | − | + | + | − | − |
| *Holosticha heterofoissneri* | + | − | − | − | − | + | + | + | − |
| *Litonotus paracygnus* | + | + | + | + | − | + | − | + | − |
| *Litonotus yinae* | ++ | ++ | ++ | + | + | + | + | + | + |
| *Loxophyllum qiuianum* | + | + | + | − | − | + | − | + | + |
| *Loxophyllum simplex* | + | + | + | + | − | + | − | + | − |
| *Metaurostylopsis salina* | ++ | ++ | +++ | + | + | ++ | + | ++ | + |
| *Orthodonella apohamatus* | + | + | ++ | ++ | + | +++ | ++++ | +++ | ++ |
| *Protocruzia contrax* | − | − | + | − | − | − | − | − | − |
| *Pseudoamphisiella elongata* | + | + | + | + | + | + | +++ | ++ | + |
| *Stephanopogon paramesnili* | ++ | − | − | + | − | − | ++++ | ++++ | + |
| *Strombidium paracalkinsi* | − | + | − | − | − | − | − | − | − |
| *Tachysoma dragescoi* | ++ | ++ | ++++ | ++ | + | +++ | ++ | ++ | + |
| *Tachysoma ovata* | + | + | + | + | − | ++ | + | − | − |

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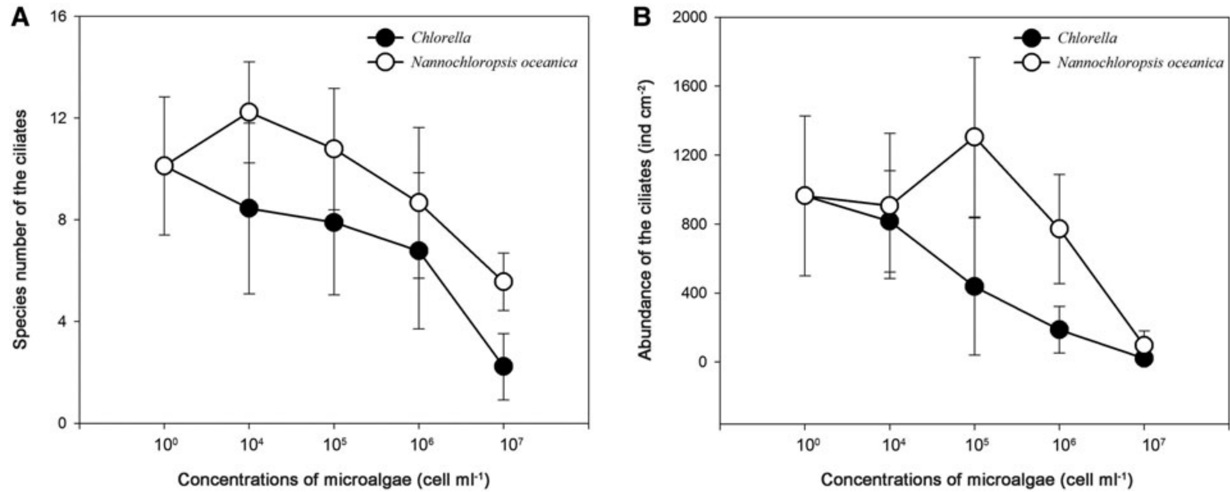


**Fig. 2.** Variations in species number (A) and abundance (B, ind. $cm^{-2}$) of protozoan communities in five treatments (1–5).

## Experimental designation

Two algal species *Nannochloropsis oceanica* and *Chlorella* sp. were used as test microalgae, which were obtained from the Laboratory of Applied Microalgae Biology, Ocean University of China.

All bioassay experiments were conducted in Petri dishes during a period of 9 days. For each of two test microalgae, five treatments with a same gradient of concentrations were designed as $10^0$ (treatment 1 as a control), $10^4$ (treatment 2), $10^5$ (treatment 3), $10^6$ (treatment 4) and $10^7$ (treatment 5) cell $ml^{-1}$, respectively. For each of both controls and a total of eight treatments, one glass slide with protozoan communities was transferred into a Petri dish with 20 ml filtered seawater (FSW) without and with test microalgae, respectively. In each treatment, two replicates were used as parallel tests.

## Identification and enumeration

Protozoa identification and enumeration were conducted following the methods outlined by Xu *et al.* (2011b). Taxonomic classification of protozoa was based on the published references such as Song *et al.* (2009). The taxonomic scheme used was according to Lynn (2008).

The enumeration of protozoa *in vivo* was conducted at a 100-fold magnification under an inverted microscope (Xu *et al.*, 2011b). For recovering all species colonizing the glass slides, the whole slide (17.5 $cm^2$) was examined to record both occurrences and individual abundances, using bright field illumination.

## Data analysis

Four taxonomic diversity/distinctness measures were summarized using four taxonomic relatedness parameters:

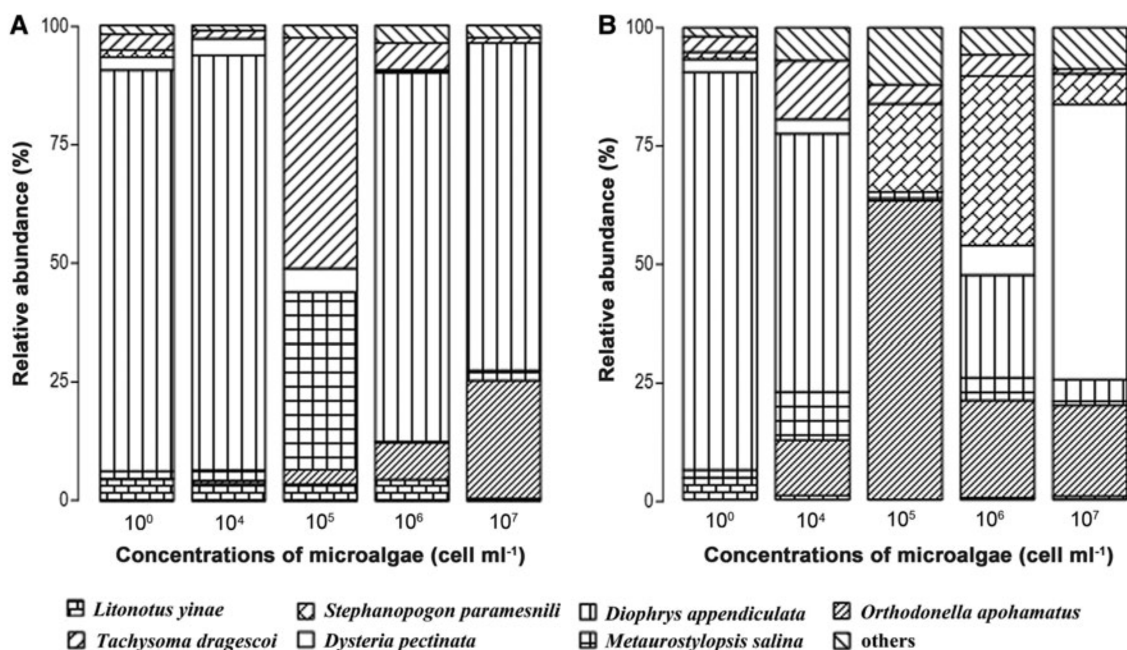


**Fig. 3.** Variations in relative species number (A) and relative abundance (B, ind. $cm^{-2}$) of protozoan communities in five treatments (1–5).

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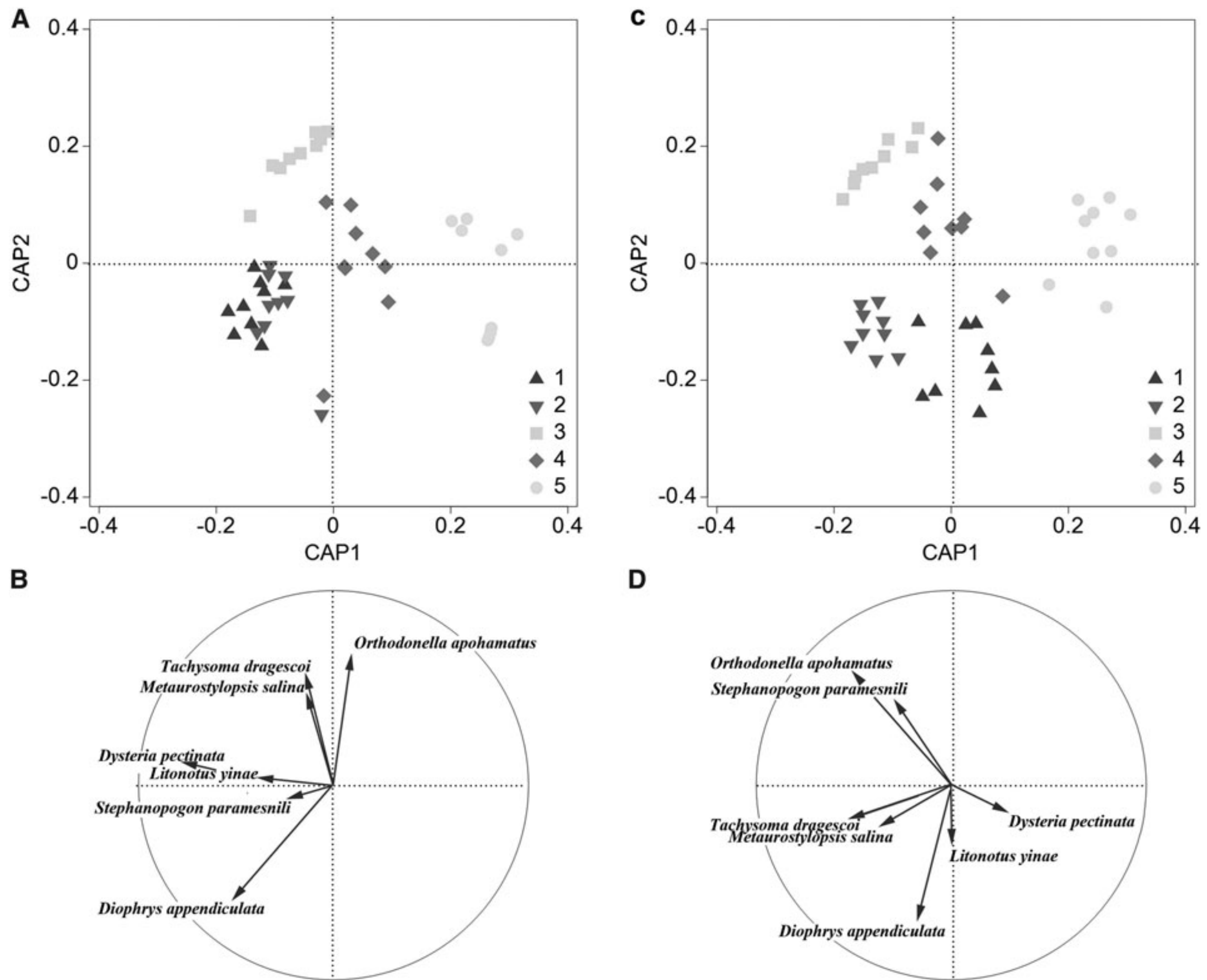


**Fig. 4.** Canonical analyses of principal coordinates (CAP) for protozoan communities (A, B), with correlations of top seven species of contributors to the protozoan communities (C, D) with the CAP axes, in five treatments (1–5) of *Chlorella* sp. (A, C) and *Nannochloropsis oceanica* (B, D).

taxonomic diversity (Δ), taxonomic distinctness (Δ*), average taxonomic distinctness ($\Delta^+$) and variation in taxonomic distinctness ($\Lambda^+$).They were computed following the equations:

$$\Delta = \frac{\Sigma\Sigma_{i<j}\omega_{ij}x_i x_j}{N(N-1)/2}$$

$$\Delta^* = \frac{\Sigma\Sigma_{i<j}\omega_{ij}x_i x_j}{\Sigma\Sigma_{i<j}x_i x_j}$$

$$\Delta^+ = \frac{\Sigma\Sigma_{i<j}\omega_{ij}}{S(S-1)/2}$$

$$\Lambda^+ = \frac{\Sigma\Sigma_{i<j}(\omega_{ij}-\Delta^+)}{S(S-1)/2}$$

where $x_i$ ($i = 1, 2, \ldots, S$) denotes the abundance of the $i$th species; $N$ is the total number of individuals in the sample; $\omega_{ij}$ is the 'distinctness weighting' given to the path length linking species $i$ and $j$ ($i < j$); $S$ is the number of species(Warwick & Clarke, 1995).

The distinctness weightings used in this study were according to Clarke & Warwick (1998): $\omega = 1$ (species in the same genus), 2 (same family but different genus), 3 (same order but different family), 4 (same class but different order) and 5 (same phylum but different class). The distinctness of two species connected at the highest taxonomic level was set equal to 100 (Warwick & Clarke, 1998, 2001). A regional master list was compiled using the data from Song *et al.* (2009), in which a total of 375 protozoa species was recorded from local areas of the Yellow Sea, near Qingdao, China.

Multivariate analyses of variations in the protozoan communities were analysed using the PRIMER v7.0.11. Bray–Curtis similarity matrices were used for biological community analysis (Xu *et al.*, 2014). The variations in protozoan community structure at five concentration levels of both algal species were summarized using the submodule CAP (canonical analysis of principal coordinates) on Bray–Curtis similarities from the transformed species-abundance data (Anderson *et al.*, 2008). Vector overlay of Pearson correlations of dominant species with the two CAP axes is also shown in CAP plots (Anderson *et al.*, 2008; Jiang *et al.*, 2011, 2013). Ellipse tests were conducted to determine the significant departure at different concentration

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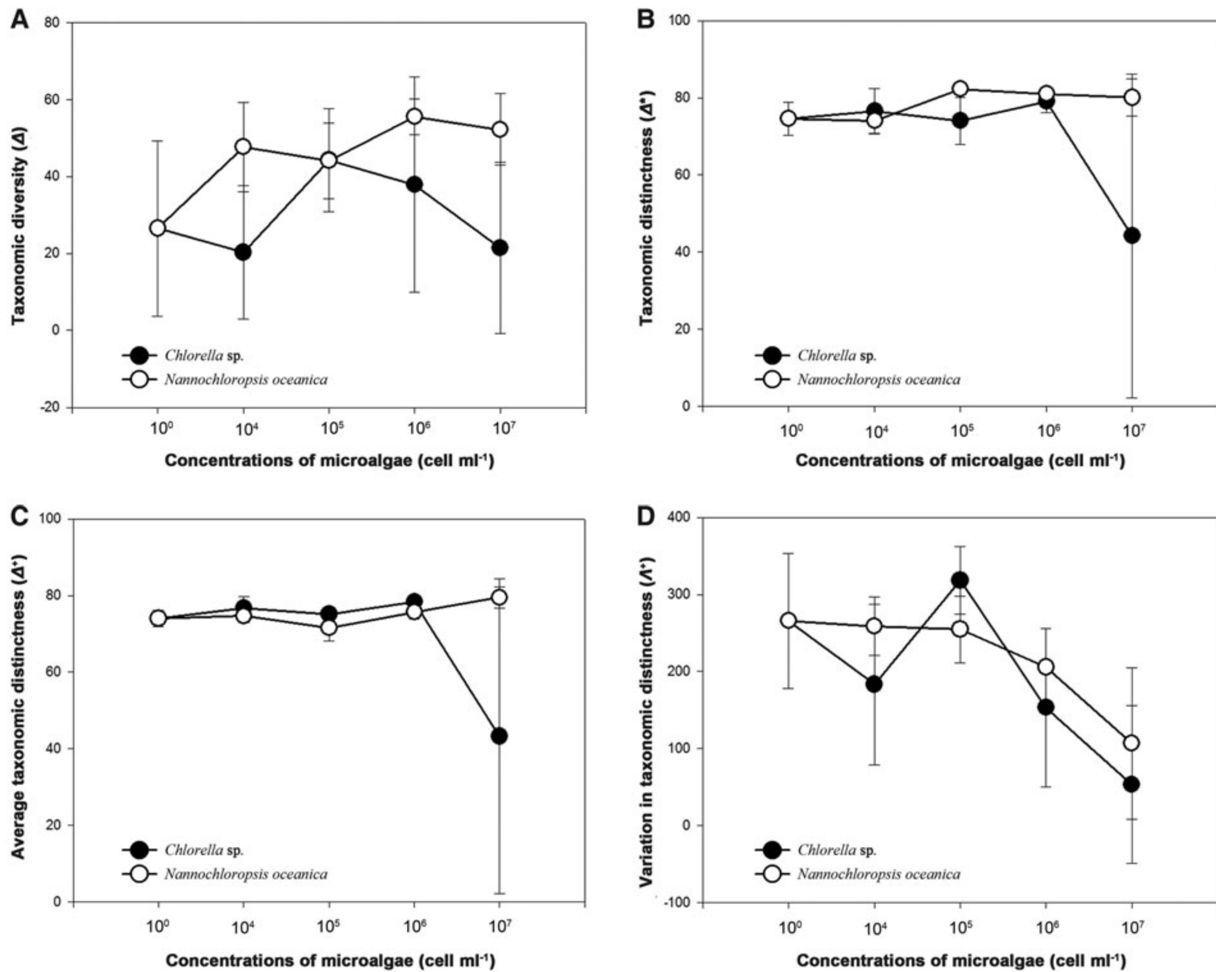


**Fig. 5.** Variations in taxonomic diversity (Δ, A), taxonomic distinctness (Δ*, B), average taxonomic distinctness ($\Delta^+$, C) and variation in taxonomic distinctness ($\Lambda^+$, D) of the protozoan communities in five treatments (1–5) of both *Chlorella* sp. and *Nannochloropsis oceanica*.

levels of the microalgae with an expected trait hierarchy using the submodule TAXDTEST (Clarke & Gorley, 2015).

## RESULTS

### Species composition of protozoan community used

The protozoa species with occurrence and abundance are presented in Table 1. In the protozoan samples used, a total of 25 protozoan species (mainly ciliates) were identified (Table 1).

### Variations in species number and abundance of protozoan samples

The species number and abundance of the protozoan samples in five treatments are shown in Figure 2. In both *N. oceanica* and *C.* sp. treatments, the minimum values of species number were recorded in treatment 5 (Figure 2A), while abundances were highest in treatment 1 and 3, and lowest in treatment 5, respectively (Figure 2B).

### Variations in community structure of protozoan samples

The relative abundance of protozoan communities at different concentration levels of two microalgae are shown in Figure 3. In treatments of *C.* sp., three structural community types of the protozoan assemblages could be recognized: (1) those dominated by *Diophrys appendiculata* and, to a lesser extent, *Litonotus yinae* (e.g. treatment 1 and 2); (2) those dominated by *Tachysoma dragescoi* and, to a lesser extent, *Metaurostylopsis salina* (treatment 3); and (3) those dominated by *Diophrys appendiculata* and, to a lesser extent, *Orthodonella apohamatus* (treatment 5) (Figure 3A).

In treatments of *N. oceanica*, five structural community types could be distinguished: (1) those dominated by *Diophrys appendiculata* and, to a lesser extent, *Litonotus yinae* (treatment 1); (2) those dominated by *Diophrys appendiculata* and, to a lesser extent, *Orthodonella apohamatus, Tachysoma dragescoi* and *Metaurostylopsis salina* (treatment 2); (3) those dominated by *Orthodonella apohamatus* and, to a lesser extent, *Stephanopogon paramesnili* (treatment 3); (4) those dominated by *Stephanopogon paramesnili* and, to a lesser extent, *Diophrys appendiculata* (treatment 4); and (5) those dominated by *Dysteria pectinata* and, to a lesser extent, *Orthodonella apohamatus* (treatment 5) (Figure 3B).

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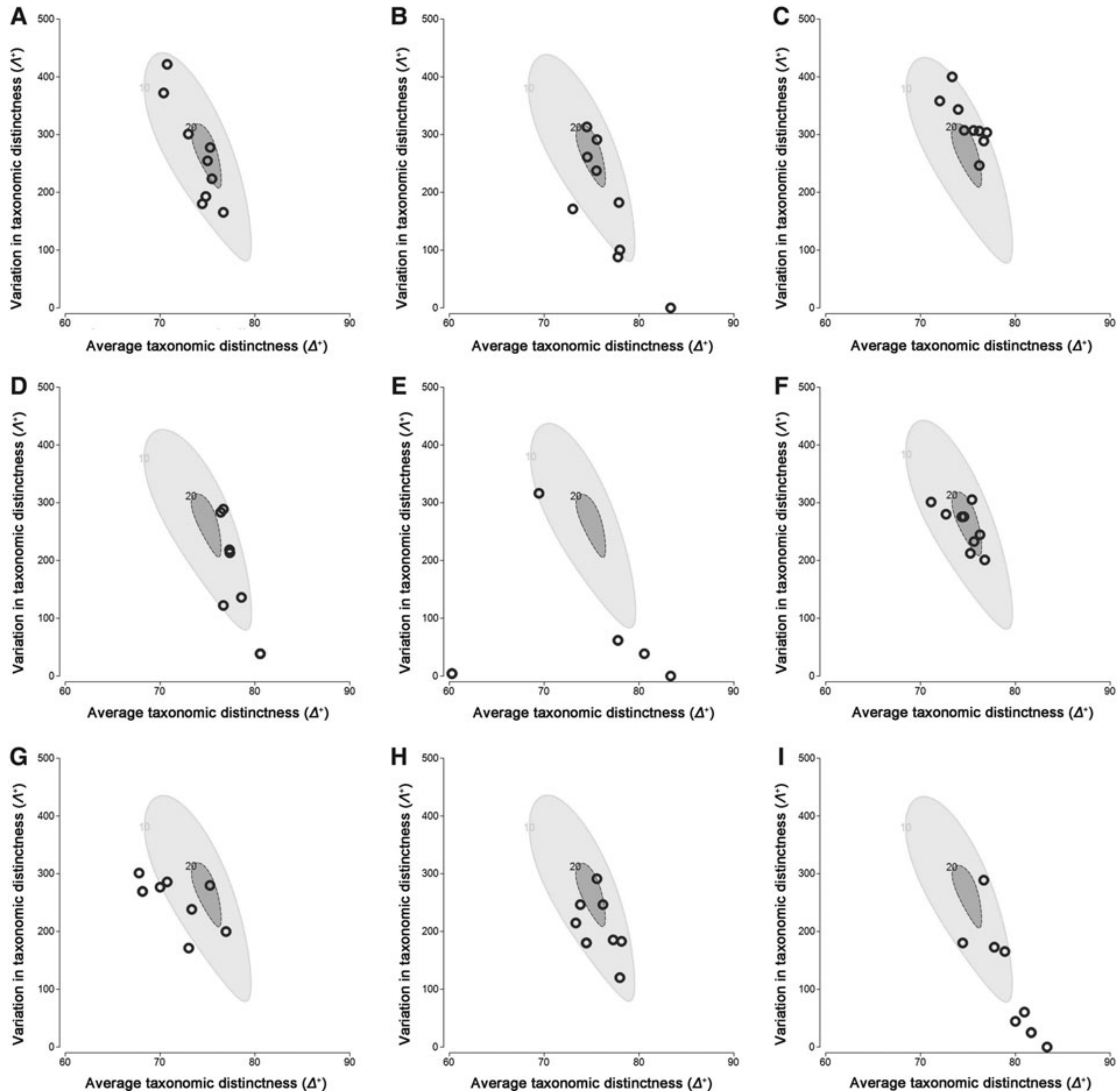


**Fig. 6.** Ellipse plots of 95% probability regions with a range of two sub-list sizes (10 and 20) for the pair-wise ($\Delta^+$, $\Lambda^+$) values of the ciliate samples in five treatments (1–5) of both *Chlorella* sp. (A–E) and *Nannochloropsis oceanica* (A, F–I), showing the departure of the protozoan samples from an expected range of 10-species sub-list.

Based on CAP ordinations, discrimination among 45 data of each treatment set of two microalgae showed a clear variation in community structure of the protozoa along the gradient of algal concentrations (Figure 4A, C). For example, in the treatments of *C.* sp., the first canonical axis (CAP 1) separated protozoan communities of treatment 5 (right side of figure) from other four treatments (left side of figure); the second canonical axis (CAP 2) discriminated data points of treatments 3 and 4 from those of treatments 1 and 2 (upper and lower parts of Figure 4A), respectively. A similar pattern was also found in the treatments of *N. oceanica* (Figure 4C).

Vector overlay of Pearson correlations of top seven typical species with the CAP axes is shown in Figure 4B, D. For example, in the treatments of *C.* sp., vectors for two species (*Diophrys appendiculata* and *Stephanopogon paramesnili*) pointed towards data points of treatments 1 and 2; five (*Orthodonella apohamatus, Metaurostylopsis salina, Dysteria pectinata, Litonotus yinae, Tachysoma dragescoi*) and vectors for the remaining three species pointed towards the datapoint clouds of treatments 3 and 4 (Figure 4B). However, a different case was found in the treatments of *N. oceanica* (Figure 4D).

## Variations in taxonomic diversity/distinctness of protozoan communities

In treatments of *C.* sp., except for the taxonomic diversity ($\Delta$), the taxonomic distinctness ($\Delta^*$) and the average taxonomic distinctness ($\Delta^+$) of the protozoan samples generally represented a sharp decrease in treatment 5, while those in treatments of *N. oceanica* levelled off within the gradient of algal concentrations (Figure 5A–C). However, the variation in taxonomic distinctness ($\Lambda^+$) generally showed a clear

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decreasing trend along the gradient of algal concentrations in treatments of both microalgae (Figure 5D).

The ellipse plots of 95% probability regions, with a range of two sub-list sizes (10 and 20) for the pair-wise ($\Delta^+$, $\Lambda^+$) values of the small species pool at all five treatments, are shown in Figure 6. It is shown that the $\Delta^+ - \Lambda^+$ data points of treatment 5 showed a different behaviour: 5 and 8 data were outside the 10 sub-size contours in treatments of *C.* sp. (Figure 6B–E) and *N. oceanica* (Figure 6F–I), respectively. It should be noted that no samples of the protozoa departed from the expected range of 10-species sub-list

## DISCUSSION

Multivariate approaches are a powerful tool to detect changes in community structure (Clarke & Ainsworth, 1993; Jiang *et al*., 2007, 2011; Xu *et al*., 2011a). In this study, CAP ordination demonstrated that the variations in protozoan community structure are related to the algal concentration gradient. A significant variation in community pattern of protozoa was found to be driven by algal concentrations, during which both species richness and abundance of protozoa decreased with an increase of algal concentrations. These findings suggest that two microalgae *N. oceanica* and *C.* sp. have strong defence effects on protozoan grazing.

Taxonomic diversity/distinctness have been widely used to summarize the internal trait of taxonomic relatedness pattern of a community (Warwick & Clarke, 1995, 2001). Compared with traditional biodiversity measures (e.g. species diversity), they have desirable properties such as less dependence on environment and sample and high sensitivity to microalgae concentration (Warwick & Clarke, 1995, 2001). In this study, the taxonomic distinctness and average taxonomic distinctness ($\Delta^*$ and $\Delta^+$) of protozoa communities represented a high sensitivity to high concentration of microalgae. Furthermore, ellipse tests demonstrated an increasing trend of departure from the expected taxonomic pattern with increase of algal concentrations. Thus, it is suggested that this approach may be used as a potential tool for identifying defence of microalgae against protozoa grazing.

In summary, both *N. oceanica* and *C.* sp. represented a significant defence effect against protozoan grazing, especially at high density levels of the algae. Species richness, abundance and taxonomic distinctness of the protozoan showed a sharp decrease at high concentration level ($10^7$ cell ml$^{-1}$) of both algae. A significant variation in community structure of the protozoa was found to be driven by the gradient of the algal concentrations. The paired biodiversity indices of the protozoan communities showed an increasing trend of departure from the expected taxonomic pattern with increase of algal concentrations. Based on the results, we suggest that the community-based bioassay might be used as a feasible tool for identifying defence against protozoan grazing of microalgae.

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