



# Population dynamics and reproduction of the heterotrophic dinoflagellate *Noctiluca scintillans* in Jiaozhou Bay, China

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**ABSTRACT:** Outbreaks of the heterotrophic dinoflagellate *Noctiluca scintillans* have impacted the Chinese coastal ecosystem for decades; however, which and how environmental factors affect its reproductive pattern and further influence its population dynamics remain to be investigated. From January 2019 to January 2020, 9 cruises were conducted at 12 stations in Jiaozhou Bay, China. The different life stages of *N. scintillans* were distinguished and quantified with the assistance of microscopic observation and quantitative real-time PCR (qRT-PCR). *N. scintillans* was seasonally abundant in winter–spring and September, but depleted in summer and October. The asexual reproduction of *N. scintillans*, which appeared as binary fission, mainly occurred in colder water (<11°C), and could be the major mode of reproduction driving population growth in March–April. In September, even though the asexual mode is by this time restricted by high water temperatures (>26°C), *N. scintillans* blooms still occurred with the assistance of sexual reproduction and the potential coupling effect of a favorable food source and physical environment, in which chain-forming diatoms were dominant in the phytoplankton community and gametes could be accumulated by continuous mild southeast winds and rainless weather. The present study contributes to a better understanding of the life cycle of *N. scintillans* in natural seawaters and provides valuable information to assess the variation in the abundance of gametes.

**KEY WORDS:** *Noctiluca scintillans* · Dinoflagellate · Population dynamics · Reproduction · Environmental factors

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## 1. INTRODUCTION

The dinoflagellate *Noctiluca scintillans* (Macartney) Kofoid & Swezy, 1921, one of the most common red tide forming organisms, impacts coastal ecosystems through oxygen depletion (Huang & Qi 1997) and potential ammonium toxicity (Morton & Twentyman 1971, Elbrächter & Qi 1998, Baliarsingh et al. 2016) during

massive blooms. This heterotrophic dinoflagellate has 2 forms differentiated by nutrition mode: red *Noctiluca* and green *Noctiluca*. Red *Noctiluca* is broadly distributed in temperate and sub-tropical regions with a lethal temperature of ~27°C and is completely phagotrophic (Elbrächter & Qi 1998, Harrison et al. 2011, Tsai et al. 2018). A wide variety of food items have been reported, including phytoplankton, cope-

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pods and their eggs, fish eggs, fecal pellets of marine organisms, and possibly bacteria (Hattori 1962, Nakamura 1998, Kiørboe 2003, Zhang et al. 2017, Tsai et al. 2018), while phytoplankton, in particular diatoms, are considered to be the main food resource in natural seawater (Dela-Cruz et al. 2002, Zhang et al. 2017, Tsai et al. 2018). Green *Noctiluca* can be phagotrophic, but it can survive when relying solely on its photosynthetic symbiont *Pedinomonas noctilucae* (Sweeney 1976, Hansen et al. 2004, Furuya et al. 2006, Saito et al. 2006). This type is mainly limited to 25–30°C in some tropical waters (Harrison et al. 2011).

Both asexual and sexual reproduction is present in the 2 forms of *N. scintillans*. Asexual reproduction is conducted by binary fission, whereas sexual reproduction involves meiosis, which forms gametogenic cells that can release 256–1024 mature haploid gametes and then results in diploid zygotes via gamete fusion (Zingmark 1970, Elbrächter & Qi 1998, Fukuda & Endoh 2006). The sexual reproduction of *N. scintillans* has been proposed previously in field research by counting the proportion of gametogenic cells under the light microscope (Dela-Cruz et al. 2003, Sriwoon et al. 2008, Zhang et al. 2017). While gametes were tiny (10–15 µm in diameter), fragile, and morphologically similar to typical dinoflagellates (Elbrächter & Qi 1998, Fukuda & Endoh 2006), during field investigations, it was hard to assess the abundance of gametes due to the difficulty of sample preservation and morphological discrimination. With the development of modern molecular biology, the quantitative real-time PCR (qRT-PCR) approach, which enables species-specific detection and quantification of particular target species, has been successfully applied in the field research of marine dinoflagellates (Galluzzi et al. 2004, Moorthi et al. 2006, Yuan et al. 2012) and therefore provides an available option for gamete enumeration in the field research of *N. scintillans*.

Asexual reproduction is considered the major mode of population growth and is more closely associated with population dynamics in the field (Zhou & Wu 1994, Uhlig 1995), while how sexual reproduction contributes to *N. scintillans* populations is not clear. Fukuda & Endoh (2006) captured the process of gamete fusion and zygote development in laboratory observations and filled the cognitive gap between sexual reproduction and population growth. Gametogenic cells of green *Noctiluca* were frequently observed in the rapid growth phase in coastal waters of Thailand. In Sagami Bay, Japan, numerous gametes occurred before or during the peak of *N. scintillans* abundance and were detectable even when vegeta-

tive cells were absent from field observations (Miyaguchi et al. 2008). It was suggested that gametes might promote population growth through zygote development (Fukuda & Endoh 2006), or reset the process in which vegetative cells transform into gametogenic cells after 22–24 divisions (Sato et al. 1998). These prior studies suggest that the contribution of sexual reproduction should be further elucidated considering its significance in genetic evolution and population growth.

Asexual reproduction of *N. scintillans* showed diurnal rhythms and was easily affected by water temperature and nutritional conditions (Uhlig 1995, Huang et al. 1997, Elbrächter & Qi 1998, Tian et al. 2017), whereas the factors impacting sexual reproduction are as yet undetermined. Sato et al. (1998) suggested that the formation of gametogenic cells follows a strict sequence of events, which starts to operate in every cell after a defined number of cell cleavages, and is mainly stimulated by the positive and negative feedback regulation mechanisms in the population, while seasonal variations might affect the occurrence of gametogenic cells in red *Noctiluca* (Fukuda & Endoh 2006). In a previous study, the feeding preference of red *Noctiluca* for chain-forming diatoms was confirmed by comparing phytoplankton community structures of field water before and after ingestion, in which the ratios of dinoflagellates to diatoms (0.13) and unchained to chained diatoms (0.30) in the experimental group were higher than in the control group (0.09 and 0.21), and it was speculated that sexual reproduction might respond earlier and faster than asexual reproduction when sufficient food was present (Tian 2017). The important role of phagotrophy was also emphasized in the sexual reproduction of green *Noctiluca* (Sriwoon et al. 2008); however, which biotic and abiotic factors affect this process, and how, is still unknown.

Jiaozhou Bay is a semi-enclosed coastal bay connecting to the Yellow Sea, and has been heavily impacted by various anthropogenic activities derived from the rapid development of Qingdao, China, as well as by subsequent harmful algal blooms (HABs) in the past decades (Wu et al. 2005, Liu et al. 2009, Yuan et al. 2016). Red *Noctiluca* is one of the common HAB species in the coastal waters of China and has frequently caused large-scale blooms (Elbrächter & Qi 1998, Harrison et al. 2011, Song et al. 2016). For example, a massive *N. scintillans* red tide caused severe damage to bivalve aquaculture in 2000 in Jiaozhou Bay (Wu et al. 2005). No green *Noctiluca* was observed along the Chinese coast, and there was also low genetic variation in the various strains of red

*Noctiluca* (Pan et al. 2016), despite the minor difference in thermal adaptability (Huang & Qi 1997, Wang et al. 2018). Previous studies paid more attention to the abundance dynamics and ecological roles of *N. scintillans* populations. The dynamics of its population structure have been rarely studied, and particularly the variation of gametes in field waters has seldom been reported. Gametes play an important role in *N. scintillans* populations as a part of the life cycle, and abundance as a general parameter needs to be included in population studies.

Thus, to provide a basic understanding of the contribution of both sexual and asexual reproduction to the population dynamics of red *Noctiluca*, a 1 yr comprehensive field survey was conducted in Jiaozhou Bay, China. The abundance of the different life stages of *N. scintillans* and their associations with major environmental factors were assessed systemically.

## 2. MATERIALS AND METHODS

### 2.1. Study area and cruises

Jiaozhou Bay, a partly-enclosed waterbody, is located on the west coast of the South Yellow Sea (Fig. 1). Its area is approximately 390 km<sup>2</sup>, with an average depth of 7 m. Water exchange between the

bay and the open water is driven by a semi-diurnal tide through a narrow channel. Strong turbulent mixing induced by tides results in nearly homogeneous vertical profiles of temperature and salinity, thus the stratification is weak even in summer (Z. Liu et al. 2004). The study area belongs to the temperate monsoon climate zone, with an average water temperature of ~15°C (Zhang et al. 2019). The prevailing wind is from the southeast in spring and summer, and from the northwest in autumn and winter.

Nine cruises were conducted using RV ‘Chuangxin’ from January 2019 to January 2020 (months are indicated in Fig. S1 in the Supplement, at [www.int-res.com/articles/suppl/m693p055\\_supp.pdf](http://www.int-res.com/articles/suppl/m693p055_supp.pdf)). During each cruise, 12 stations were sampled in the inner, mouth, and outer areas of Jiaozhou Bay (Fig. 1). Sampling was only conducted in the daytime and finished within 2 or 3 d (dates are given in Fig. S1).

### 2.2. Environmental sampling and data collection

Water temperature and salinity were determined by shipborne CTD probes (AAQ1183-1F, Alec Electronics). Cells of *Noctiluca scintillans* were gathered by towing a conical plankton net vertically (mesh size = 160 µm, mouth aperture = 0.08 m<sup>2</sup>) from near the bottom to the surface. The net samples were pre-

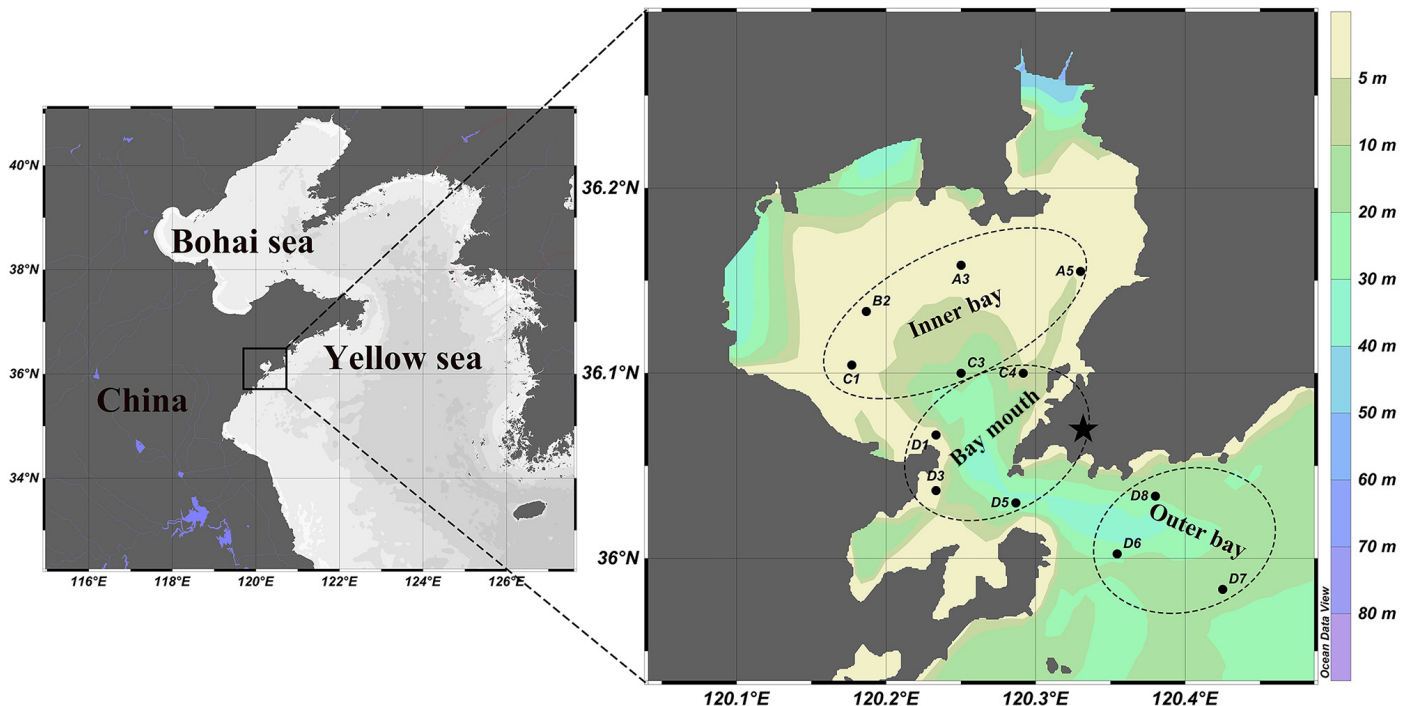


Fig. 1. Locations of sampling stations in Jiaozhou Bay, China. The 12 stations were divided into 3 groups: inner bay, bay mouth, and outer bay (distinguished by ellipses). The star symbol shows the location of the meteorological station

served immediately with borate-buffered formaldehyde (final concentration: 5 %). A flow meter (Hydro-Bios) was mounted at the mouth of the plankton net to measure the volume of water filtered according to the 'Specifications for oceanographic survey Part 6: Marine biological survey' (GB/T 12763.6-2007, <https://www.chinesestandard.net/PDF/English.aspx/GBT12763.6-2007>). Seawater samples for nutrients, chlorophyll *a* (chl *a*), phytoplankton, and *N. scintillans* gametes were collected from the surface, middle, and bottom layers using Niskin bottles. Inorganic nutrients (NO<sub>3</sub>-N, NO<sub>2</sub>-N, NH<sub>4</sub>-N, PO<sub>4</sub>-P, and Si(OH)<sub>4</sub>-Si) were measured using an autoanalyzer (San++ system, Skalar), and chl *a* concentrations were determined using a fluorometer (Trilogy, Turner) according to Strickland & Parsons (1972). Phytoplankton samples were concentrated as described by Utermöhl (1958), and were then identified and counted under an inverted microscope (IX 71, Olympus).

The precipitation and wind vector data were obtained from the data set 'SURF\_CLI\_CHN\_MUL\_DAY, V3.0' of the China Meteorological Data Service Center (station code: 54857, latitude: 36° 04' 00" N, longitude: 120° 20' 00" E, altitude: 76 m, see <http://data.cma.cn>) (Fig. 1).

### 2.3. Observation and quantification of *N. scintillans* cells

Net samples of *N. scintillans* were enriched to 50 ml, and then observed under a stereomicroscope (SZ61, Olympus). Vegetative cells, dividing cells, and gametogenic cells were identified as described by Fukuda & Endoh (2006) and further enumerated, and the diameters of vegetative cells were measured using the Cellsens Software with which the Olympus DP80 was equipped.

To detect and quantify gametes in the water column of each station, equal volumes of seawater from all layers were mixed to a final volume of 2 l and filtered through a 20 µm sieve to remove vegetative cells. The water sample was then filtered onto a 3 µm polycarbonate membrane (47 mm diameter, Millipore).

Genomic DNA was extracted from the membranes using the DNeasy PowerSoil® Kit (Qiagen). The amplification was based on a 167 bp fragment from the 18S and internal transcribed spacer 1 (ITS1) region of the *N. scintillans* ribosomal RNA (rRNA) gene complex, using a species-specific primer set (forward primer: 5'-GAA GTC GTA ACA AGG TTT CCG TAG-3'; reverse primer: 5'-GCG AAC TTT GCT GAC CGT AA-3') designed utilizing Primer Premier 5.0. qRT-PCR was per-

formed on a Rotor-Gene Q 2plex HRM thermocycler (Qiagen) in a 20 µl reaction mixture, which was composed of 1 µl of genomic DNA, 1 µl (10 µM) of each primer, 10 µl of SYBR Premix Ex *Taq* II (2 ×) (TaKaRa), and 7 µl of DNAase/RNAase-free water. PCR amplification conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 30 s, after which an additional melting curve was generated for the specificity test.

To construct a standard curve to calculate the abundance of gametes, a DNA sample of pure gametes developed in the laboratory was serially diluted, corresponding to a range of 100–16 000 cells per reaction. The regression equation for cells was  $y = -3.4373x + 29.0458$  ( $R^2 = 0.9937$ ), where  $x$  indicates the denary logarithm of cell numbers ( $\lg N_{\text{cell}}$ ), and  $y$  indicates the cycle threshold ( $C_t$ ) value. Additionally, serial dilutions of plasmid DNA containing the target sequence ( $10^9$  to  $10^3$  copies per reaction) were assessed to investigate the average copy number of the target sequence per cell. The regression equation for recombinant plasmids was  $y = -3.4416x + 37.9792$  ( $R^2 = 0.9996$ ), where  $x$  indicates the denary logarithm of plasmid copy numbers ( $\lg N_{\text{plasmid}}$ ), and  $y$  is the  $C_t$  value.

### 2.4. Statistical analysis

To evaluate the chl *a*, nutrients, and phytoplankton communities in water columns, depth-averaged values were calculated based on the discrete data from the surface to the bottom. The dissolved inorganic nitrogen (DIN) is the sum of NO<sub>3</sub>-N, NO<sub>2</sub>-N, and NH<sub>4</sub>-N, dissolved inorganic phosphorus (DIP) is PO<sub>4</sub>-P, and the dissolved silicic acid is Si(OH)<sub>4</sub>-Si.

Statistical analyses were conducted with SPSS 19.0 for Windows. Normality and homogeneity of variance were checked by the Kolmogorov-Smirnov test and Levene's test, respectively, both at a significance level ( $\alpha$ ) of 0.05. Kruskal-Wallis tests were performed to analyze the differences in environmental factors, phytoplankton communities, and *N. scintillans* parameters (vegetative cells, dividing cells, gametogenic cells, gametes, and cell diameters) among the sampling months. Furthermore, multiple comparisons were conducted when the test indicated significant differences ( $p < 0.05$ ). Mann-Whitney *U*-tests were performed to determine the differences between abundances and proportions of dividing cells and gametogenic cells every month, with  $\alpha = 0.05$ .

The Spearman correlation coefficient was applied to calculate correlations between different types of *N. scintillans* cells and environmental factors using



the 'corrplot' package (v.0.84) in the R computing environment, with the false discovery rate adjusted significance level of FDR adjusted  $P$  set at 0.05.

### 3. RESULTS

#### 3.1. Environmental characteristics

The environmental parameters indicated overt temporal patterns over the sampling months ( $p < 0.05$ , Kruskal-Wallis test, Table S1). Water temperature fluctuated between 2.7°C (January 2019) and 28.6°C (September). Salinities were relatively stable in all sampling months, with a range of 30.2–33.0, but values in the inner bay were significantly lower ( $p < 0.01$ , Kruskal-Wallis test). Chl *a* was relatively high (4.1–6.6  $\mu\text{g l}^{-1}$ ) in particular stations of the inner and mouth area in June (Stns A3, A5, B2, C1, C3, C4, see Fig. 1) and September (Stns A5, C1, C4), with maximum and minimum occurring in June (6.6  $\mu\text{g l}^{-1}$ ) and December (0.5  $\mu\text{g l}^{-1}$ ), respectively. DIN ranged from 2.9  $\mu\text{M}$  (July) to 44.8  $\mu\text{M}$  (October), DIP ranged from 0.3  $\mu\text{M}$  (June) to 1.6  $\mu\text{M}$  (October), and dissolved silicic acid ranged from 0.9  $\mu\text{M}$  (December) to 16.7  $\mu\text{M}$  (October). The concentrations of DIN, DIP, and dissolved silicic acid were generally lower in spring or summer, and peaked in autumn, especially in October. Other than the fact that the inner bay area was distinguished by higher DIN, there was no significant difference in the nutrient concentrations of the 3 areas ( $p > 0.05$ , Kruskal-Wallis test).

Daily precipitations and wind vectors are shown in Fig. S1. Except for a rainfall that occurred on 10 October and resulted in 13.2 mm of precipitation, all samplings were conducted in rainless conditions. There was no significant difference in the *Noctiluca scintillans* population between the rainless day (8 October) and the rainy day (10 October) ( $p > 0.05$ , Mann-Whitney  $U$ -test). Northwest wind prevailed from mid-to-late September to January, with a velocity range of 1.0–8.5  $\text{m s}^{-1}$  (mean = 3.4  $\text{m s}^{-1}$ ). Southeast winds prevailed during the other periods, with a velocity range of 1.1–7.0  $\text{m s}^{-1}$  (mean = 3.1  $\text{m s}^{-1}$ ).

#### 3.2. Variation in the phytoplankton community

A total of 156 phytoplankton species (in 69 genera) were identified in Jiaozhou Bay during the sampling period. The cell abundance of major phytoplankton species varied by 3 orders of magnitude (0.4–524.8 cells  $\text{ml}^{-1}$ ), including 2 distinct peaks in April–June

and September (Fig. 2A). Bacillariophyta and Dinophyta were the major groups in the phytoplankton community, with *Chaetoceros* sp., *Skeletonema* sp. (March–June), *Pseudo-nitzschia delicatissima*, *C. curvisetus*, *Thalassiosira* sp., *Nitzschia* sp., and *T. rotula* being the most dominant species in each sampling month. The detailed temporal distribution of the 32 genera with proportions exceeding 1% is shown in a heat map (Fig. 2B) in which *Thalassiosira*, *Skeletonema*, and *Chaetoceros* exerted the highest dominance in the bay. *Thalassiosira* was observed throughout the sampling months, maintaining a high and stable proportion of 14.4–29.9% (except for July and September). *Skeletonema* was mostly dominant from late winter to early summer, contributing more than 80% of the cell abundance in April, and supporting the phytoplankton community in June together with *Cerataulina* and *Thalassiosira*. The rise in abundance in September was mainly due to the extremely high abundance of phytoplankton at Stns A5, C1, and C4, to which *Chaetoceros* contributed most.

To further assess the correlation between *N. scintillans* and the phytoplankton community, the phytoplankton community was further divided into 4 functional groups: 'unchained diatoms' (49 species), 'chained diatoms' (47 species), 'dinoflagellates' (57 species), and 'others' (1 Dictyophyceae and 2 Chlorophyta), based on feeding preference (Tian 2017). The average proportions of these groups in each sampling month are shown in Fig. 2C. Chained diatoms were most abundant from January to April in 2019, accounting for at least 85.7% of the plankton community. Such dominance was particularly evident in April with an average proportion of 97.5% (94.2–99.6%). The proportion of chained diatoms gradually decreased in summer, while the proportion of dinoflagellates increased dramatically from 23.8% (June) to 43.8% (July). In autumn, abundant chained diatoms, which were mainly composed of *C. curvisetus*, prevailed briefly in September, accounting for more than 90% of the plankton community at Stns A3, A5, C1, and C4, then decreased in the next month. From late autumn to late winter, the proportion of chained diatoms increased from 48.2% (October 2019) to 66.0% (January 2020), implying the recovery of their dominance.

#### 3.3. Dynamics of the *N. scintillans* population and its association with environmental variables

The population of *N. scintillans* was highly associated with seasonal patterns ( $p > 0.05$ , Kruskal-

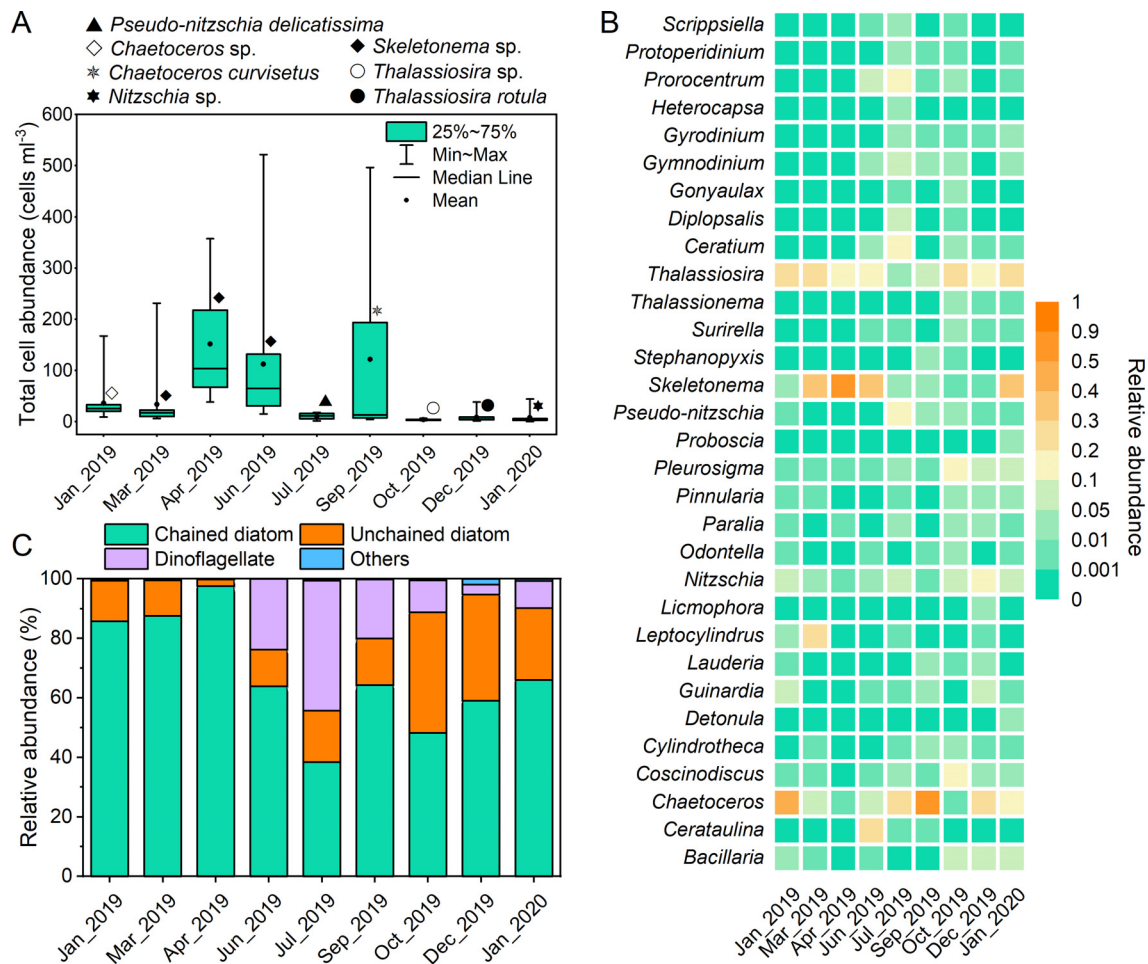


Fig. 2. Variations in (A) phytoplankton abundance, (B) the composition of phytoplankton communities at the genus level, and (C) the composition of phytoplankton communities at the functional group level in the sampling months

Wallis test), but not with the geographical location of the sampling stations ( $p < 0.05$ , Kruskal-Wallis test), ranging from no occurrence in the samples (June) to a maximum of  $2.8 \times 10^4$  cells  $m^{-3}$  (September) (Fig. 3). The first peak of the *N. scintillans* population was observed in spring, with the highest abundance of  $5.7 \times 10^3$  cells  $m^{-3}$  in April, implying its recovery from the previous winter. Another peak in the *N. scintillans* population appeared and reached the annual maximum in September. The proportion of fed *N. scintillans* cells (i.e. cells with prey in their vacuoles) was commonly high in January 2019 and March ( $>80\%$ ), decreased in April (23.3–93.3%), continued to decrease in June and July (20–55%), had a brief recovery in September ( $\sim 60\%$ ), and finally increased to over 80% in December and January 2020 (Fig. S2). However, many of the fed cells could not be identified because the contents were almost digested, and the recognizable prey were mainly diatoms, including *Coscinodiscus*,

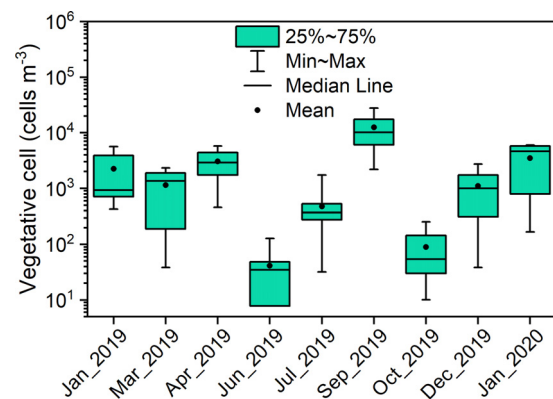


Fig. 3. Variation in the abundance of *Noctiluca scintillans* vegetative cells across sampling months

*Odontella*, *Paralia*, *Thalassiosira*, and some broken frustules.

Cell diameters were used to represent reproductive vitality (Dela-Cruz et al. 2003). The diameters of

*N. scintillans* cells ranged from 210.2 to 1223.0  $\mu\text{m}$ , and the size distribution varied significantly in different months ( $p < 0.05$ , Kruskal-Wallis test) (Fig. 4A,B). Cells with diameters within the range of 450–550  $\mu\text{m}$  occupied the highest proportion (~25%), and this range was set as the cutoff to discriminate larger and smaller cells in the present study (Fig. 4B). In the spring months, the populations were mainly dominated by larger *N. scintillans* cells ( $>550 \mu\text{m}$ ), and most cells had larger cell diameters than in other months. For other sampling periods, distribution approximated a bell curve (Fig. 4C), and mean diameters were close to the cutoff range (Fig. 4A).

The *N. scintillans* population was highly correlated with the phytoplankton community, exhibiting

a negative correlation with the chl *a* concentration ( $p < 0.05$ , Spearman correlation analysis, Table 1), and a strong relationship with the proportion of chained diatoms ( $p < 0.01$ ). In particular, *N. scintillans* abundance increased every time chained diatoms prevailed. Within the population, the 3 kinds of reproductive cells all showed a positive correlation with the abundance of vegetative cells ( $p < 0.01$ ). The proportion of fed cells showed significant positive correlations with water temperature, salinity,  $\text{Si(OH)}_4\text{-Si}$ , and the proportion of chained diatoms ( $p < 0.01$ , Spearman correlation analysis, Table 1), and was negatively correlated with the proportion of unchained diatoms and dinoflagellates ( $p < 0.05$ ,  $p < 0.01$ ).

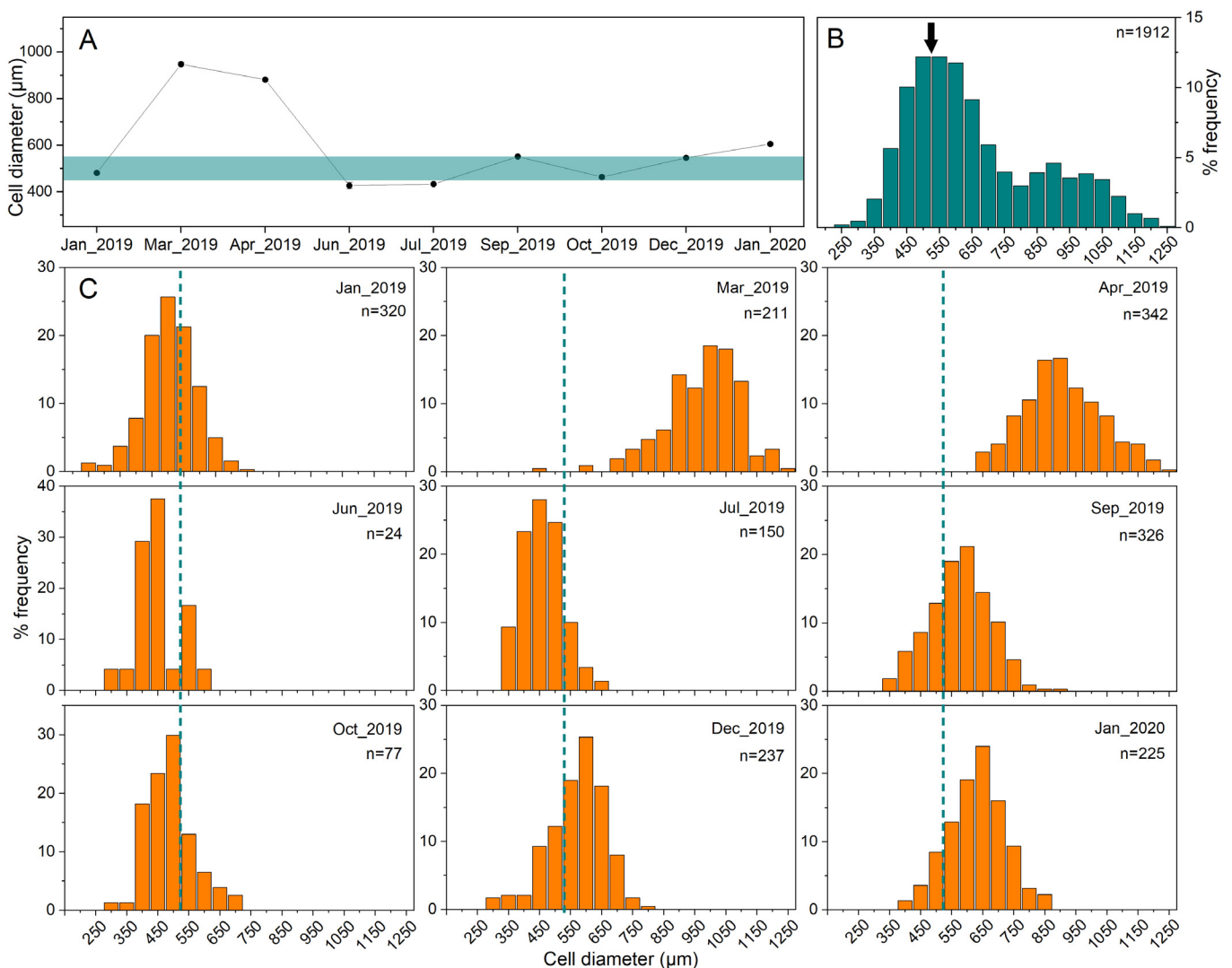


Fig. 4. (A) Cell diameters (mean  $\pm$  SE) of *Noctiluca scintillans* determined from 9 sampling months. (B) Total frequency distribution of cell diameters measured from 9 sampling months; cell diameters were categorized into 50  $\mu\text{m}$  size class intervals, ranging from 150 to 1250  $\mu\text{m}$ . (C) Detailed frequency distributions of cell diameters measured in the respective sampling months. The green rectangle in (A), the arrow in (B), and dashed lines in (C) indicate the modal cell diameter range (450–550  $\mu\text{m}$ )

Table 1. Spearman correlation coefficients between different types of *Noctiluca scintillans* cells and major environmental factors. Significant values are highlighted in **bold** (\* $p < 0.05$ , \*\* $p < 0.01$ ). DIN: dissolved inorganic nitrogen; DIP: dissolved inorganic phosphorus; fed cells: cells with prey in their vacuoles

	Vegetative cells	% Dividing cells	% Gametogenic cells	Gametes	% Fed cells
Temperature	-0.063	<b>-0.608**</b>	-0.200	0.167	<b>-0.604**</b>
Salinity	<b>0.231*</b>	-0.091	0.101	0.068	<b>-0.372**</b>
Chl <i>a</i>	<b>-0.235*</b>	-0.102	-0.074	-0.212	-0.077
DIN	-0.152	0.174	0.053	-0.025	-0.039
DIP	0.009	0.067	0.055	0.196	0.044
Si(OH) <sub>4</sub> -Si	-0.173	<b>-0.348**</b>	-0.125	0.185	<b>-0.541**</b>
Phytoplankton	0.045	0.182	0.189	-0.152	-0.227
% Unchained diatoms	<b>-0.247**</b>	<b>-0.234*</b>	<b>-0.333**</b>	-0.018	<b>-0.223*</b>
% Chained diatoms	<b>0.308**</b>	<b>0.433**</b>	<b>0.407**</b>	0.018	<b>0.382**</b>
% Dinoflagellates	-0.120	<b>-0.507**</b>	<b>-0.312**</b>	0.081	<b>-0.296**</b>
% Others	0.128	<b>-0.215*</b>	0.005	0.248	0.191

### 3.4. Reproductive cells and their associations with environmental factors

Dividing cells were commonly observed in January 2019, March, April, and in January 2020. These cells were still detectable at a few stations (at most 6 sampling stations each month) in July, September, and December, but completely disappeared in the June and October sampling. The abundances of dividing cells ranged from 0 to 121.3 cells  $m^{-3}$ , with the highest proportion of 20% dividing cells in the total amount of *N. scintillans* cells (Fig. 5A; Fig. S3A). Gametogenic cells were generally observed in January 2019, April, September, December, and January 2020, with a range of 0–45.8 cells  $m^{-3}$  and a proportion of up to 1.9% in the total amount of *N. scintillans* cells (Fig. 5B; Fig. S3B). In January 2019, March, and January 2020, dividing cells represented 50–100, 50–100, and 57.1–100% (except 38.1% at Stn D6) of the reproductive cells, respectively; the proportions in the total amount of *N. scintillans* cells were significantly higher than gametogenic cells ( $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.05$ ; Mann-Whitney *U*-test). In September, gametogenic cells represented 66.7–100% of the reproductive cells except at Stns A5 (45.5%) and C1 (33.3%), and the proportions in the total amount of *N. scintillans* cells were significantly higher than the dividing cells ( $p < 0.01$ , Mann-Whitney *U*-test). The variation in gamete abundance was coincident with the vegetative cell abundance most of the time, with the minimum occurring in June ( $1.1 \times 10^4$  to  $6.4 \times 10^6$  cells  $m^{-3}$ ) and the maximum occurring in September ( $1.8 \times 10^7$  to  $2.7 \times 10^8$  cells  $m^{-3}$ ). However, during winter–spring, gametes were more abundant in January 2019 and 2020 ( $1.1 \times 10^6$  to  $1.6 \times 10^8$  cells  $m^{-3}$ ) (Fig. 5C), while vegetative cell abundance peaked in April (Fig. 3).

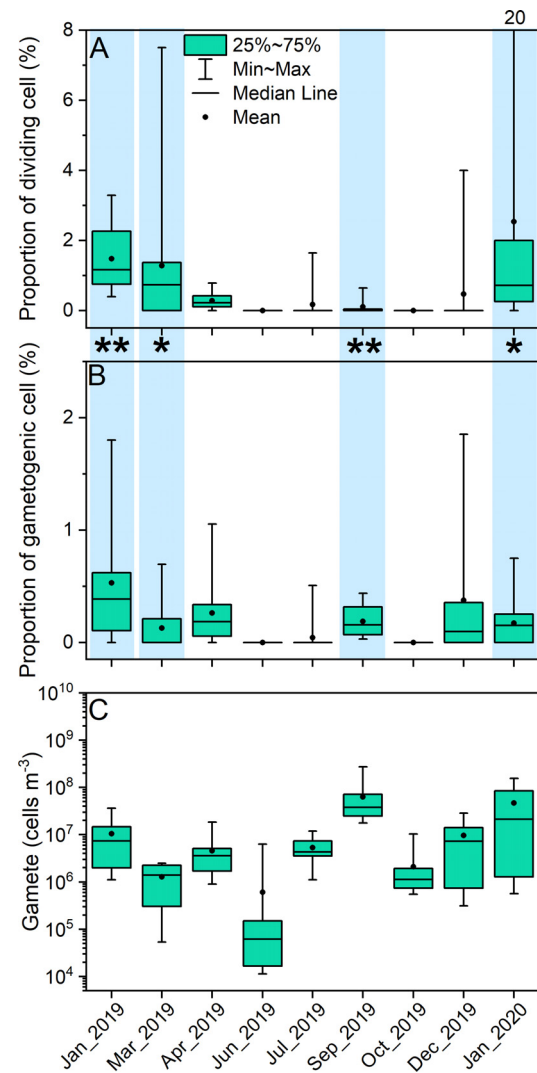


Fig. 5. Variations in (A) the proportion of dividing cells, (B) the proportion of gametogenic cells, and (C) the abundance of gametes. Asterisks and blue rectangles in (A) and (B) indicate significant differences between dividing cells and gametogenic cells (\* $p < 0.05$ ; \*\* $p < 0.01$ , Mann-Whitney *U*-test)



Both the dividing cells and the gametogenic cells exhibited a positive relationship with the proportion of chained diatoms ( $p < 0.01$ ), and were commonly observed when chained diatoms dominated the phytoplankton community (Fig. 6C,D), whereas the abundance of gametes showed no significant correlation with the proportion of chained diatoms ( $p > 0.05$ ). Dividing cells were highly restrained by increasing water temperature (Fig. 7C). As indicated by Spearman correlation coefficient analyses, the proportion of dividing cells was negatively associated with seawater temperature ( $p < 0.01$ ), while gametogenic cells and gametes showed no relationship with water temperature ( $p > 0.05$ ). Within the population, the 3 types of reproductive cells were correlated with each other ( $p < 0.01$ ), except for the non-significant correlation between dividing cells and gametes ( $p > 0.05$ ). There was a significant positive correlation between the proportions of dividing cells and gametogenic cells and the proportion of fed cells in the total amount of *N. scintillans* cells ( $p < 0.01$ ) (Table S2).

#### 4. DISCUSSION

Asexual reproduction has always been considered the dominant reproduction mode for the growth of the *Noctiluca scintillans* populations (Zhou & Wu 1994, Uhlig 1995), while its sexual reproduction is more complicated, involving multiple life stages that are hard to distinguish (Fukuda & Endoh 2006), and thus not well understood. In particular, gametes, as a vital component of the *N. scintillans* population, were previously always absent from field investigation reports due to the unavailability of reliable detection methods. In the present study, vegetative cells, dividing cells, gametogenic cells, and gametes of *N. scintillans* were quantitatively assessed to reveal the population dynamics and reproduction patterns in Jiaozhou Bay, China. The results suggest that both asexual and sexual reproduction of *N. scintillans* are able to maintain population growth and show seasonal variance by the coupling effect of biological (favorable food source), hydrographical (water temperature), and meteorological conditions (wind and rainfall).

##### 4.1. Population dynamics of *N. scintillans*

The abundance of vegetative cells varied seasonally during the sampling period (Fig. 3), but there was no clear pattern of spatial distribution in Jiaozhou Bay, which may result from the frequent water exchanges

between the inner and outer bay (Wang et al. 2018). In Jiaozhou Bay, the cell abundances of *N. scintillans* were normally below 100 cells  $l^{-1}$  (Wang et al. 2018) and the abundance (0–27 cells  $l^{-1}$ ) measured in the present study was within the common range. The appearance of its first peak was consistent with previous observations in the bay (Wang et al. 2018), which was also observed in many other temperate coastal waters (Murray & Suthers 1999, Tada et al. 2004, Mikaelyan et al. 2014). In Dapeng Bay (located in southern China), *N. scintillans* was generally abundant within a temperature range of 17.4–24.5°C (Huang & Qi 1997), whereas in Jiaozhou Bay (northern China), higher cell abundances ( $> 25$  cells  $l^{-1}$ ) usually occurred when seawater temperatures ranged from 3.4 to 15.0°C (Wang et al. 2018). The second peak abundance of *N. scintillans* occurred in September when the water temperature ( $> 26^{\circ}C$ ) was no longer suitable for its population growth in Jiaozhou Bay (Wang et al. 2018). Such a peak of *N. scintillans* in late summer and autumn has also been observed in Sagami Bay, Japan (Miyaguchi et al. 2008), and the coastal waters of south-eastern Australia (Murray & Suthers 1999).

##### 4.2. Ecological roles of asexual and sexual reproduction in population growth of *N. scintillans*

The proportions of both dividing cells and gametogenic cells were positively correlated with the abundance of vegetative cells (Table S2). There was no doubt that the 2 reproductive types could maintain the growth of the *N. scintillans* population, even when it is still uncertain how sexual reproduction plays a role. Sato et al. (1998) suggested that gametes could induce vegetative cells to conduct binary fission in *N. scintillans*, thereby resulting in a population increase. Active cell division supported by numerous gametes might be an indispensable part of red tides (Miyaguchi et al. 2006). Microscopic observation of *N. scintillans* gamete fusion and zygote development indicated that gametes could contribute directly to population growth by forming new vegetative cells (Fukuda & Endoh 2006). In the present study, gametes showed no correlation with dividing cells (Table S2). Moreover, when gametes reached peak abundance in September (up to  $2.7 \times 10^8$  cells  $m^{-3}$ ), dividing cells almost disappeared in the water column (0–0.6%). All results indicated that the sexual reproduction of *N. scintillans* could contribute to population growth by forming zygotes.

Both asexual and sexual reproduction played important roles in the population growth of *N. scintil-*

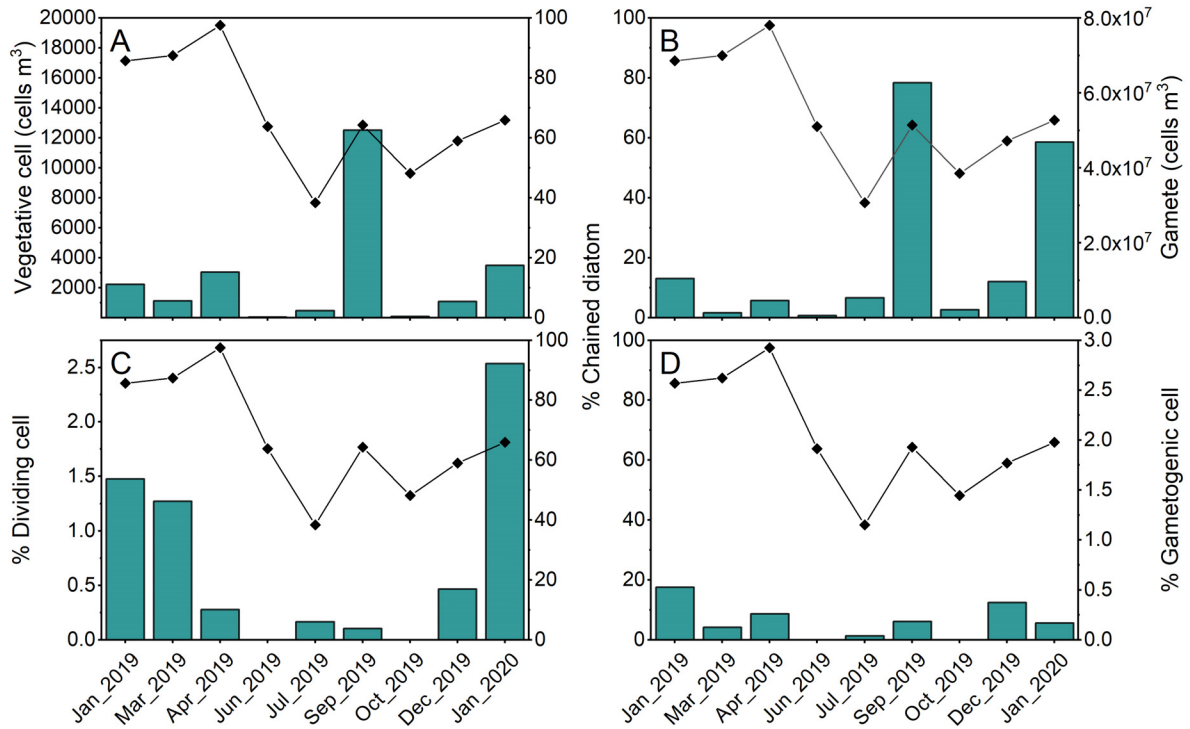


Fig. 6. Averages (indicated by columns) of (A) the abundance of vegetative cells, (B) the abundance of gametes, (C) the proportion of dividing cells, and (D) the proportion of gametogenic cells, and their associations with fluctuations in the proportion of chained diatoms in the phytoplankton community (indicated by lines) determined from each sampling month

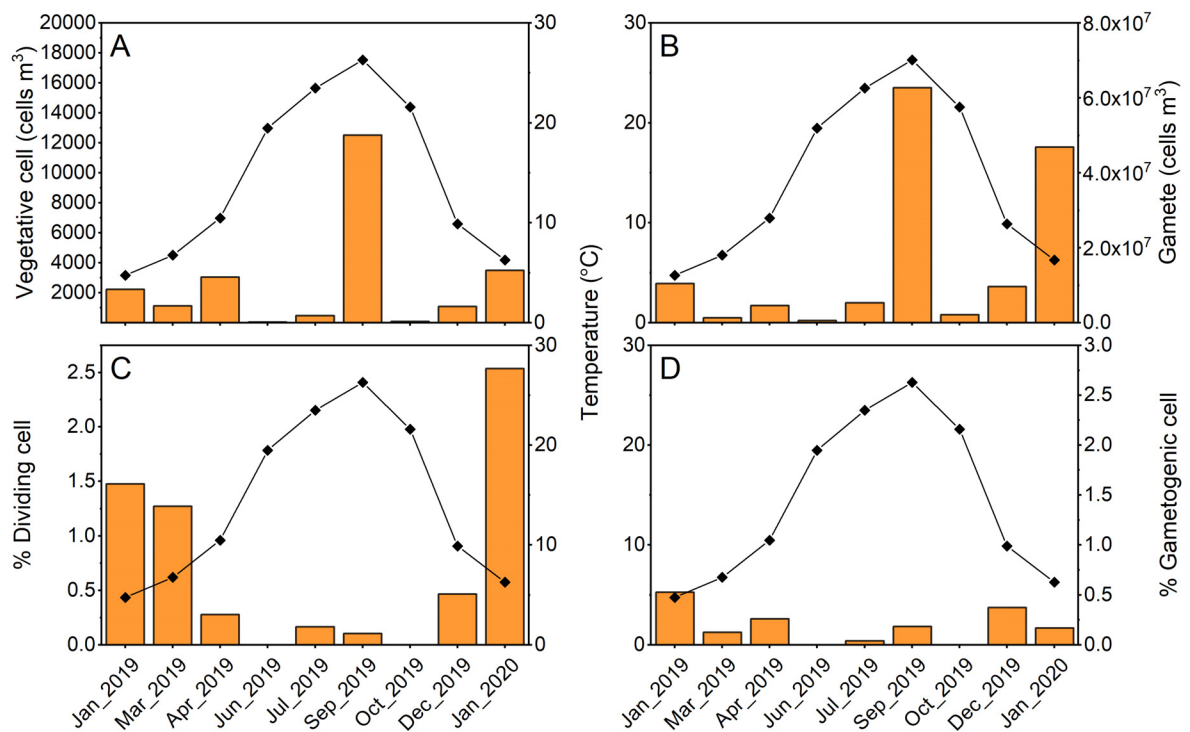


Fig. 7. Averages (indicated by columns) of (A) the abundance of vegetative cells, (B) the abundance of gametes, (C) the proportion of dividing cells, and (D) the proportion of gametogenic cells, and their associations with fluctuating water temperatures (indicated by lines) in different sampling months

*lans*. In previous studies, the peak of binary fission typically appeared in spring when dividing cells take up a large proportion of *N. scintillans* populations, and is closely associated with population growth (Zhou & Wu 1994, Uhlig 1995, Dela-Cruz et al. 2002). In the present study, active asexual reproduction started in the early winter, continued to maintain higher proportions (>50%) in the reproductive cells in January–March, then decreased until the next winter, which was coincident with the appearance of the winter–spring peak (Fig. 5A). The abundances of *N. scintillans* were low in June, July, and October, and dividing cells were barely detectable in the water column, which in turn implied the inactiveness of cellular cleavage during that period. When the cell abundance of *N. scintillans* approached its second peak in September, the gametogenic cells accounted for higher proportions (>66.7%) of the reproductive cells, implying an important contribution of sexual reproduction to this uncommon peak. The variation in the abundance of gametes could reflect the dynamics of sexual reproduction in *N. scintillans* populations. Similar to the observations of Miyaguchi et al. (2008), the abundances of gametes and vegetative cells were highly correlated in the present study (Table S2), which emphasized the potential importance of sexual reproduction in population growth. Moreover, the gamete abundance could provide more detailed information when gametogenic cells cannot be observed in June, July, and October. Vegetative cell abundances were relatively low in these 3 months, while gametes were still detectable and even with comparable abundances in July and October (up to  $1.2 \times 10^7$  cells  $m^{-3}$ ) as in March and April (up to  $1.8 \times 10^7$  cells  $m^{-3}$ ) (Fig. 5C), suggesting that gametes might serve as seeds to trigger *N. scintillans* blooms in favorable environments.

The field data showed that few small cells (<450  $\mu m$  in diameter) of *N. scintillans* were present in March and April (Fig. 4C). The zygotes of *N. scintillans* were approximately 15–25  $\mu m$  in diameter immediately after gamete fusion, went through a series of morphologic changes to develop into small vegetative cells with diameters of ~150  $\mu m$ , then continued to grow into mature vegetative cells (>200  $\mu m$  in diameter) (Zingmark 1970, Fukuda & Endoh 2006). The whole process in green *Noctiluca* takes a few days, during which the cell diameter is intermediate between 30 and 170  $\mu m$  (Lirdwitayaprasit 2001). Thus, the size distribution of *N. scintillans* cells in March–April, when large cells accounted for a major proportion of the *N. scintillans* populations, indicated the limited contribution of developing zygotes to growth,

and further suggested the populations were mainly maintained by binary fission. However, due to the lack of direct observation evidence, the current study could not confirm the existence of zygotes based only on the cell diameter distribution.

#### 4.3. Effects of major factors on reproduction of *N. scintillans*

Both the active asexual and sexual reproduction of red *Noctiluca* are thought to rely on sufficient nutrition support, as the vegetative cell loses all of its feeding organs during the formation of reproductive cells (Elbrächter & Qi 1998, Fukuda & Endoh 2006). Green *Noctiluca* also forms gametogenic cells when there is an adequate external food supply (Sriwong et al. 2008). In the present study, chl *a* exhibited no clear correlation with all types of reproductive cells but was negatively related to the vegetative cells. Such a relationship might reflect the higher predation pressure of *N. scintillans* (up to  $2.8 \times 10^8$  cells  $m^{-3}$ ) on other phytoplankton (Huang & Qi 1997). In the present study, the highest abundance of *N. scintillans* was  $2.8 \times 10^3$  cells  $m^{-3}$ , and was not able to exert much pressure on the prey. There may be other factors that inhibited population growth even though the food supply was sufficient, leading to the situation in which the high abundance of *N. scintillans* did not coincide with high chl *a* concentration.

Chained diatoms (e.g. *Thalassiosira* spp., *Pseudo-nitzschia* spp.) have frequently been detected in the water column as well as in the food vacuoles of *N. scintillans* (Dela-Cruz et al. 2002, McLeod et al. 2012, Tsai et al. 2018, Zhang et al. 2021), and were confirmed to be the favored prey for *N. scintillans* in an earlier study (Tian 2017, Text S1, Figs. S4 & S5). In the present study, both sexual and asexual reproduction of *N. scintillans* were highly correlated with chained diatoms ( $p < 0.01$ ); the increasing abundance of *N. scintillans* population was coincident with the dominance of chained diatoms (Fig. 6), then decreased when dinoflagellates or unchained diatoms became more dominant. Moreover, the high proportion (~80%) of fed cells was also coincident with the increasing proportion of chained diatoms ( $p < 0.01$ ), and had a brief recovery (~60%) when chained diatoms prevailed again in September, implying that suitable phytoplankton community structure could promote the reproduction of *N. scintillans*.

Temperature played a key role in affecting the seasonal variation of *N. scintillans* reproduction in Jiaozhou Bay. Active binary fission usually occurred in

the colder water of winter and spring, and high proportions of dividing cells have been detected in water temperatures as low as 3–6°C (Uhlir 1995, Tian et al. 2017). Asexual reproduction was greatly inhibited when water temperature exceeded 25°C (Huang et al. 1997, Tian et al. 2017). In the present study, the proportion of dividing cells exhibited a significantly negative relationship with water temperature, and the sampling months with the proportion of dividing cells exceeding 1% were mainly restricted to spring and winter when the water temperature was under ~11°C. Tian et al. (2017) found that there was a brief recovery of sexual reproduction in September, which was also observed in the present study. Both the proportion of gametogenic cells and the abundance of gametes were not closely associated with the increasing seawater temperature. Further, when the water temperature peaked in September, gametogenic cells accounted for higher proportions in the reproductive cells, together with the highest abundance of gametes in the water column. In summary, asexual reproduction was more active in relatively cold water (<11°C), while sexual reproduction might contribute more to population growth when the seawater becomes warmer.

Buoyant *N. scintillans* cells are easily driven by winds and currents, and the development and dispersion of algal blooms are susceptible to fluctuations in the physical environment (Mikaelyan et al. 2014, S. Zhang et al. 2017, W. Zhang et al. 2020). The highest abundance of *N. scintillans* was observed in September when the water temperature was not stimulative to active cell division (Fig. 7A). Similarly, in south-eastern Australia, an outbreak of *N. scintillans* occurred in late summer, which was probably promoted by the physical accumulation from surrounding waters (Murray & Suthers 1999). Currents could be the potential vector for the migration of *N. scintillans* cells (McLeod et al. 2012). The wind-driven surface currents induced aggregation of *N. scintillans* cells in forming high biomass in Port Shelter, and the tidal motions refreshed the nutrient supply for better growth of prey items (*Chaetoceros curvisetus*, *Skeletonema costatum*, and *Eucampia zodiacus*) in Jiaozhou Bay, which were likely to play important roles in forming *N. scintillans* blooms (Yin 2003, D. Liu et al. 2004, Zhao et al. 2011, Lai & Yin 2014, Zhang et al. 2017). Moreover, although the present study did not show the effect of rainfall because almost all samplings were conducted in rainless conditions, precipitation may also contribute to the collapse of *N. scintillans* blooms by affecting the properties and stability of the water, while stable

humid weather without heavy rain is considered to be another important factor for bloom-forming events (Huang & Qi 1997, Zhang et al. 2021). It is possible that the stable southeastern winds and rainless weather provided favorable conditions for transportation and aggregation of buoyant algal cells, which might bring seeds of *N. scintillans* from the open sea to Jiaozhou Bay. Meanwhile, with the assistance of wind-driven surface currents, numerous gametes might accumulate and greatly increase the rate of zygote formation, and the population of *N. scintillans* could be maintained in September even with inactive asexual reproduction.

#### 4.4. Limitation and future directions

How sexual reproduction contributes to the population growth of *N. scintillans* in natural seawater and which environmental factors play a vital role in triggering this mode of reproduction are key questions to understand the life strategy and bloom mechanism of *N. scintillans*. The current study provided speculation based on monthly field data that need to be supported by quantitative descriptions. The sexual reproduction of *N. scintillans* consists of a series of morphologic and genetic changes including gamete formation, gamete release, zygote formation, and zygote development (Zingmark 1970, Elbrächter & Qi 1998, Fukuda & Endoh 2006).

In the present study, we included the abundance of gametes in a field investigation of the population dynamics of *N. scintillans* for the first time. In addition, cell diameter distribution was used to find evidence in support of the occurrence of zygote development in seawater. However, the diameter of developing zygotes was intermediate between gametes (10–15 µm) and vegetative cells (>200 µm), thus we were not able to confirm the occurrence of zygotes due to the loss of most intermediate cells by vertical towing (mesh size = 160 µm). Due to the low fusion rate of *N. scintillans* (1–2‰) under laboratory conditions (Zhou & Wu 1994), it is not possible to acquire enough pure zygotes to generate a standard curve, thus the current qRT-PCR approach to detect gametes cannot be applied to detect and quantify zygotes in the water column. However, catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) is capable of detecting environmental microorganisms without the establishment of pure cultures (Tujula et al. 2006, Kubota 2013), and thus could be a valid technique to monitor the occurrence of *N. scintillans* zygotes. In addition, follow-up laboratory experi-



ments need to be carried out with a denser sampling regime, in which the respective growth rates contributed by the 2 modes of reproduction, the fusion rate of gametes and its correlation with gamete density, the survival rate of zygotes, and key environmental factors need to be assessed, to determine the contribution of sexual reproduction quantitatively.

## 5. CONCLUSIONS

Both asexual and sexual reproductions play important roles in maintaining the population growth of *N. scintillans* in the typical temperate coastal bay. Biological (favorable food source), hydrographical (water temperature), and meteorological conditions (wind and rainfall) influenced the reproduction pattern of *N. scintillans*, thereby further impacting the population dynamics. Asexual reproduction of *N. scintillans* was commonly observed in colder water (<11°C), and played a more important role in driving its population growth in March–April. *N. scintillans* blooms still occurred even though the asexual mode was restricted by high water temperature (>26°C). The assistance of sexual reproduction and the potential coupling effect of favorable food sources and physical environment could be the key to this process, in which the dominant chain-forming diatoms facilitate the formation of gametogenic cells, and gametes could be accumulated by continuous mild southeast winds and rainless weather.

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