

Seasonal variations of group-specific phytoplankton cell death in Xiamen Bay, China*

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Abstract The importance of phytoplankton cell death is being increasingly recognized, however, there are still no published reports on this in Xiamen Bay. In this study, the proportion of dead phytoplankton cells associated with environmental factors was assessed at a station in Xiamen Bay from December 2012 to December 2013, using a cell digestion assay, which is an effective method to analyze dead/living cells in complex natural phytoplankton communities. The percentages of dead cells (% DC) in the total phytoplankton in summer ($16\% \pm 6\%$) were lower than those in winter ($27\% \pm 16\%$). Six groups of phytoplankton (G1–G6) were categorized by flow cytometry. These phytoplankton communities with diverse seasonal variations in % DC had different responses to environmental constraints. The main factors affecting mortality were temperature and salinity, while nutrient concentration showed little influence on phytoplankton death. Additionally, our results provide evidence that chlorophyll *a* concentrations had an inverse relationship with total phytoplankton % DC and viable cell abundance was more meaningful than total cells to explain variations in environmental parameters (such as Chl *a*). Moreover, the lowest mean % DC in total phytoplankton was $16\% \pm 6\%$ at our sample site, which is in a subtropical area with high water temperatures, full solar radiation, and rich nutrients. This indicates that phytoplankton cell death is a process that cannot be ignored. In summary, phytoplankton cell death is important in understanding the dynamics of phytoplankton communities and the functioning of subtropical ecosystems.

Keyword: cell death; cell digestion assay; phytoplankton; Xiamen Bay

1 INTRODUCTION

Phytoplankton play an important role in aquatic biogeochemical cycles. They not only serve as the basis of aquatic food webs and are responsible for nearly half of all global annual carbon-based primary productivity, but they also directly influence their environment and the organisms within it. This is despite phytoplankton only accounting for <1% of the earth's biomass (Redfield, 1958; Falkowski et al., 1998; Field et al., 1998; Geider et al., 2001). A high phytoplankton production/biomass ratio indicates extremely high turnover rates, demonstrating that phytoplankton grow, die, and are replaced once a week on average (Valiela, 1995). Until the mid-1970s, studies of aquatic biogeochemical cycles were based

on the misconception that phytoplankton were immortal without grazing and sinking to balance phytoplankton growth (Liao, 2010). However, phytoplankton cell death has been observed in laboratory algal cultures. Not only is a slight decline in total cell abundance often noted after transferring the cells into fresh medium, but more obvious declines are observed after having reached a maximum abundance, clearly indicating significant cell death (Veldhuis and

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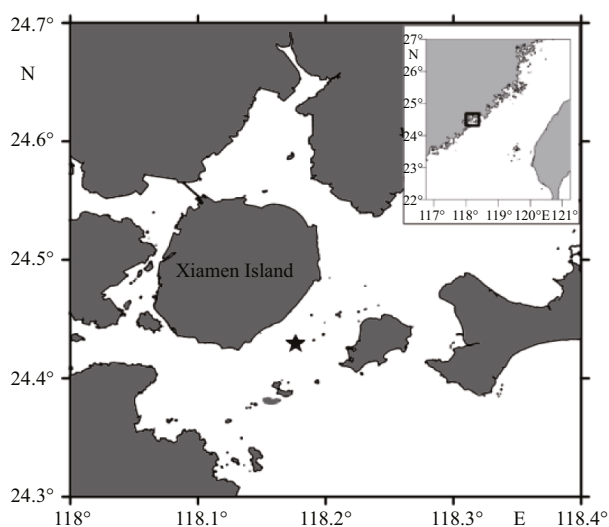


Fig.1 The sampling site in Xiamen Bay, China (black star)

Brussaard, 2006). Brussaard et al. (1995) concluded that cell lysis, as well as grazing and sedimentation, are structuring mechanisms for algal bloom dynamics. Cole et al. (1988) also documented that nearly 50% of global primary productivity requires extensive death and turnover independent of sinking and grazing.

It has become increasingly apparent that phytoplankton die in several ways, such as senescence, lysis by bacteria/virus, and programmed cell death (PCD). Wetzel (1995) examined the dynamics of dissolved organic matter (DOM) and particulate organic matter (POM) in lakes and suggested the existence of non-predatory death in phytoplankton. Agustí et al. (2001) found that the dissolved organic carbon (DOC) excreted from dead phytoplankton represented up to 70% of the net primary production in the unproductive areas of the eastern tropical North Atlantic. Alonso-Laita and Agustí (2006) reported that the lowest percentages of living cells observed in the oligotrophic open ocean of the subtropical NE Atlantic were 18.6% and 11.8% for nano- and micro-phytoplankton, respectively. Therefore, cell death has become recognized as a very important loss process that affects population dynamics and species succession (Agustí et al., 1998; Kirchman, 1999; Agustí, 2004; Bidle and Falkowski, 2004; Agustí et al., 2006). Moreover, explication of activity parameters (such as primary production and Chl *a*) is more meaningful if they can be related to viable cells rather than total cells (Garvey et al., 2007). Thus, phytoplankton cell death, which induces changes in phytoplankton biomass and net productivity, is significant for phytoplankton population dynamics,

aquatic ecosystems, and biogeochemical cycles (van Boekel et al., 1992; Brussaard et al., 1995; Smith et al., 1995; Evans and Wilson, 2008). Moreover, quantifying cell death is at least as important as studying marine phytoplankton growth.

Although there have been some field studies on phytoplankton cell death, such as in the Atlantic Ocean (Alonso-Laita and Agustí, 2006), Pacific Ocean (Hayakawa et al., 2008), Mediterranean Sea (Agustí and Sánchez, 2002), Arctic Ocean (Llabrés and Agustí, 2008), Antarctic (Llabrés and Agustí, 2010), seven Florida lakes (Agustí et al., 2006), and reservoirs (Rychtecký et al., 2014), there are still very few reports on this phenomenon and its influence on biogeochemical cycles in subtropical ecosystems. In the present study, a cell digestion assay (Darzynkiewicz et al., 1994; Agustí and Sánchez, 2002), which distinguishes living from dead cells, was applied to elucidate seasonal variations in phytoplankton cell death in Xiamen Bay. Therefore, the aims of our study were to understand: (i) whether phytoplankton cell death changes seasonally, (ii) whether it varies among taxa, and (iii) the relationship between cell death and major environmental factors.

2 MATERIAL AND METHOD

2.1 Sampling site

The study was conducted at biweekly or monthly intervals between December 2012 and December 2013 at a station (24°20'42.32"N, 118°11'08.15"E) in Xiamen Bay, Xiamen, Fujian Province, China (Fig.1). Surface waters temperature and salinity were measured by an onboard Ferrybox underway system (4-Jena, Germany). Surface water samples were collected with acid-cleaned Nalgene bottles and immediately transported to the laboratory (<2 hours) to avoid delays between sampling and cell mortality analysis.

2.2 Nutrients and chlorophyll *a* analysis

Nutrient samples (including nitrate, nitrite, phosphate, and silicate) were analyzed in an AA3 auto-analyzer (Bran-Lube, GmbH, Germany) using standard methods (Hansen and Koroleff, 1999). For total chlorophyll *a* (Chl *a*) concentration analysis, 100 mL of seawater was filtered onto Whatman GF/F filters (25 mm) under low vacuum (<150 mm Hg), and filters were extracted in 95% acetone at -20°C in the dark for 20 h; the fluorescence was then measured using the non-acidification method on a Laboratory

Fluorometer fluorometer (Trilogy, Turner Designs, America) following Welschmeyer (1994).

2.3 Phytoplankton mortality analysis

Phytoplankton abundances were assessed by flow cytometry (Cytosub, Cytobuoy b.v., Netherlands) with a 488-nm laser. Cells with similar optical properties were categorized into groups in Cytoclus software (Cytobuoy b.v., Netherlands). The clustering result was linked to objective factors provided by the Cytoclus software and subjective considerations such as experience of the observer without a fully objective statistical method (Dubelaar et al., 2003; Thyssen et al., 2008). The shape and amplitude of the spectrum of each cell were fully considered, not only using the basic parameters, such as the forward scattering (FWS), lateral scattering (SWS), red fluorescence (FLR), orange fluorescence (FLO), and yellow fluorescence (FLY), but also some simple mathematical model parameters of spectrum signal shape (e.g., inertia, asymmetry, fill factor). High-green fluorescent beads (1- μm diameter) were added to the samples as an internal standard to quantify cell concentration and to estimate the size of the small cells.

Total cell abundance, which included both living and dead cells, was counted in 25-mL fresh samples from the station. Each count was duplicated. The abundance of living cells (viability) was analyzed by a cell digestion assay as modified for phytoplankton cells by Agustí and Sánchez (2002). Briefly, 2.5 mL DNase I (800 $\mu\text{g}/\text{mL}$, Sigma-Aldrich) in Hanks' balanced salts (HBSS, pH 7.4, Sigma-Aldrich) was added to a 25-mL sample and incubated at 37°C for 15 min. We then added 2.5 mL Trypsin (2%, Sigma-Aldrich) in HBSS and incubated at 37°C for 30 min. At the end of the incubation, the samples were placed on ice to stop enzyme reactions, and the abundance of cells was quantified in the Cytosub, as described above. The cells remaining after the assay represented living (viable) cells because dead cells with compromised membranes were digested out by DNase I and Trypsin, and were undetectable by flow cytometry. The percentage of dead cells was calculated as the ratio between the total cell abundance minus living cells and total cell abundance.

2.4 Statistical analysis

Significant differences were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's

post-hoc test in SPSS Version 16.0 (SPSS Inc., Chicago, USA) for Windows. The relationships between the environment parameters (temperature, salinity, phosphates, nitrates, nitrite, and silicate), Chl *a*, and the phytoplankton group distribution/or percentage of dead cells were analyzed by the Pearson correlation in *R* (Team, 2013). In all cases, a value of $P < 0.05$ indicated significant differences/correlations.

3 RESULT

3.1 Environmental data

Surface water temperatures ranged from 13°C to 30°C, the lowest value was observed in January 2013. The temperatures then increased and peaked in August 2013. After this temperatures decreased to 19.3°C in November 2013 (Fig.2a). Salinity ranged from 21.6 to 31.1 (Fig.2b). The sample on 6 June 2013 was only 21.6 because of consecutive heavy rain on 3–5th June, 2013.

The nutrient concentrations were usually replete at this station. The phosphate concentrations varied from 0.645 $\mu\text{mol}/\text{L}$ to 1.952 $\mu\text{mol}/\text{L}$ with the lowest in mid-summer and the highest in either late autumn or early winter (Fig.2c). Nitrate + nitrite and silicate fluctuated between 12.293–51.443 $\mu\text{mol}/\text{L}$ and 28.271–85.739 $\mu\text{mol}/\text{L}$, respectively (Fig.2d and e). Chl *a* concentrations were between 0.784–8.102 $\mu\text{g}/\text{L}$, and the highest and lowest values occurred in summer and winter, respectively. Chl *a* increased from mid-spring and peaked in June 2013 when salinity decreased (Fig.2b and f).

3.2 Phytoplankton viability and dynamics

Six groups were distinguished by flow cytometry and were labeled G1–G6 with a rough taxonomic identification (Fig.3). A threshold of 30 was applied to the FLR pulsed signal recording larger cells. Because these cells had high red fluorescence but a low number, larger volumes were necessary to ensure accurate counting (e.g., G3, G5, and G6; Fig.3a). The G1, G2, and G4 clusters with low fluorescence but high abundance, were resolved with no FLR threshold (Fig.3b). G1 and G2 cells with low FLR, FLO, high FLO/FLR ratio, and small cell sizes were considered cyanobacteria, and G2 had a similar cytogram to that of a pure *Synechococcus* culture. G3 cells was identified as cryptophytes, based on the high FLR, FLO, and FLO/FLR ratio. G4–G6 had the same FLO/FLR ratio and different cell sizes, the G6 group had the biggest cells (Fig.3).

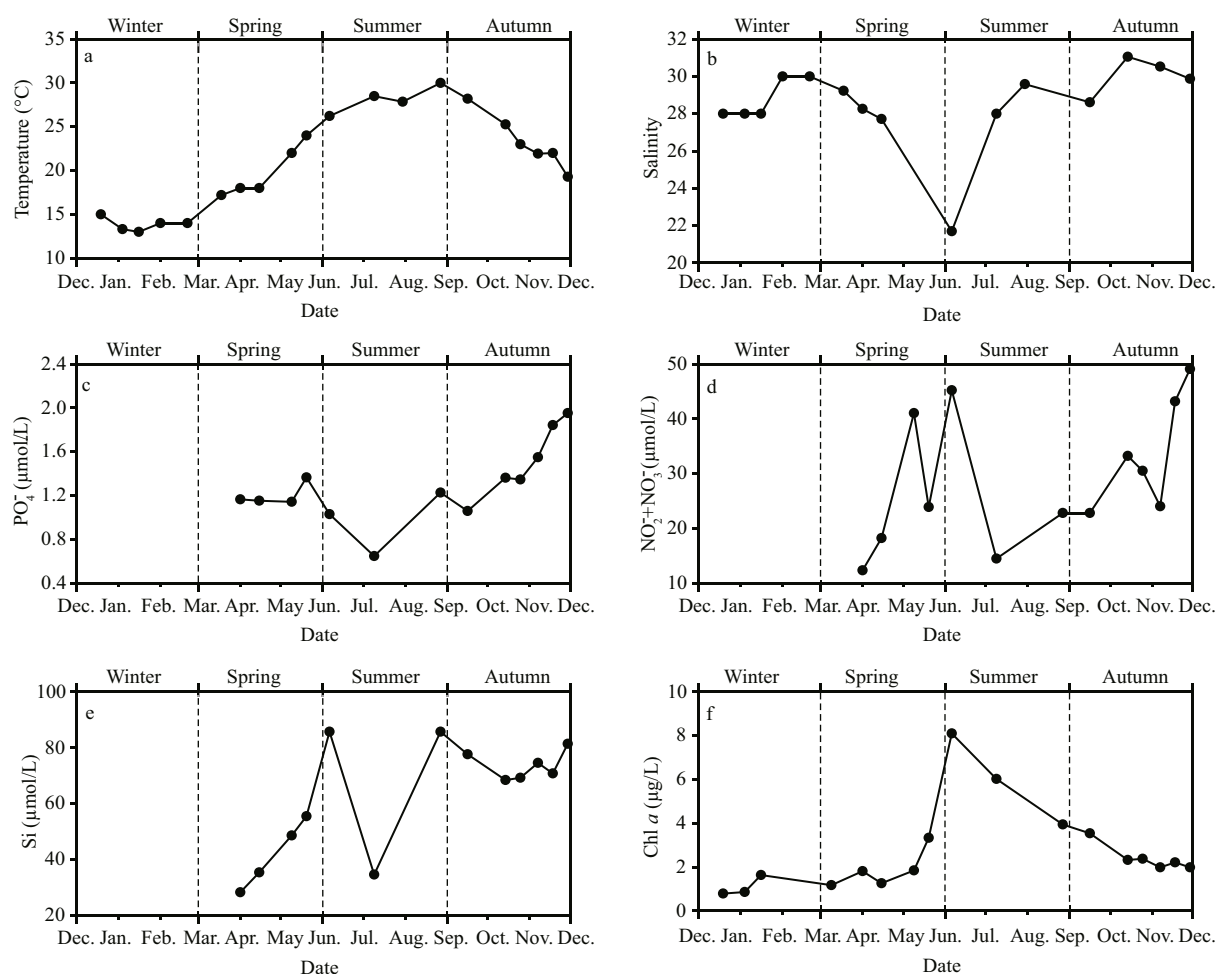


Fig.2 Environmental and biological features at the sampling site (surface water)

Temperature (a), salinity (b), PO_4^{3-} (c), $\text{NO}_2^- + \text{NO}_3^-$ (d), Si (e), and Chl *a* (f) from December 2012 to December 2013.

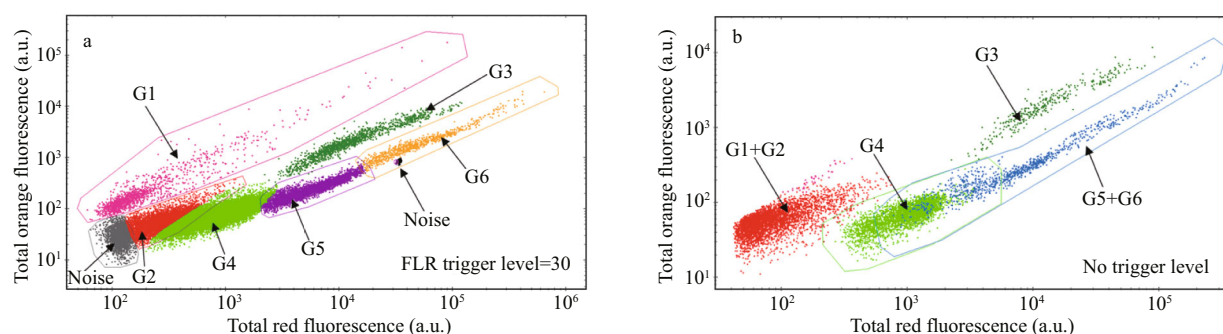


Fig.3 Red fluorescence (FLR) versus orange fluorescence (FLO) cytograms leading to the resolution of clusters (a.u.: arbitrary units)

a. a threshold on FLR recording was set at 30; b. no threshold on FLR recording.

The living cells (LC) in the six groups exhibited broadly similar seasonal patterns in total cell abundance (AC, living+dead cells), except for G3 in which LC abundance differed significantly (ANOVA, $P < 0.05$) to that of AC (Fig.4). All groups exhibited distinct seasonal variations in cell abundance throughout the year. G2 was the most abundant phytoplankton group,

reaching its peak (2.32×10^5 cell/mL) in October 2013. It accounted for 33%–81% of total phytoplankton abundance and its contribution to total cells was high in winter (on average 61%) and autumn (on average 66%), and low in summer (on average 38%; Fig.5). G1 displayed higher orange fluorescence than G2 (Fig.3), and was less abundant, it also reached maximum

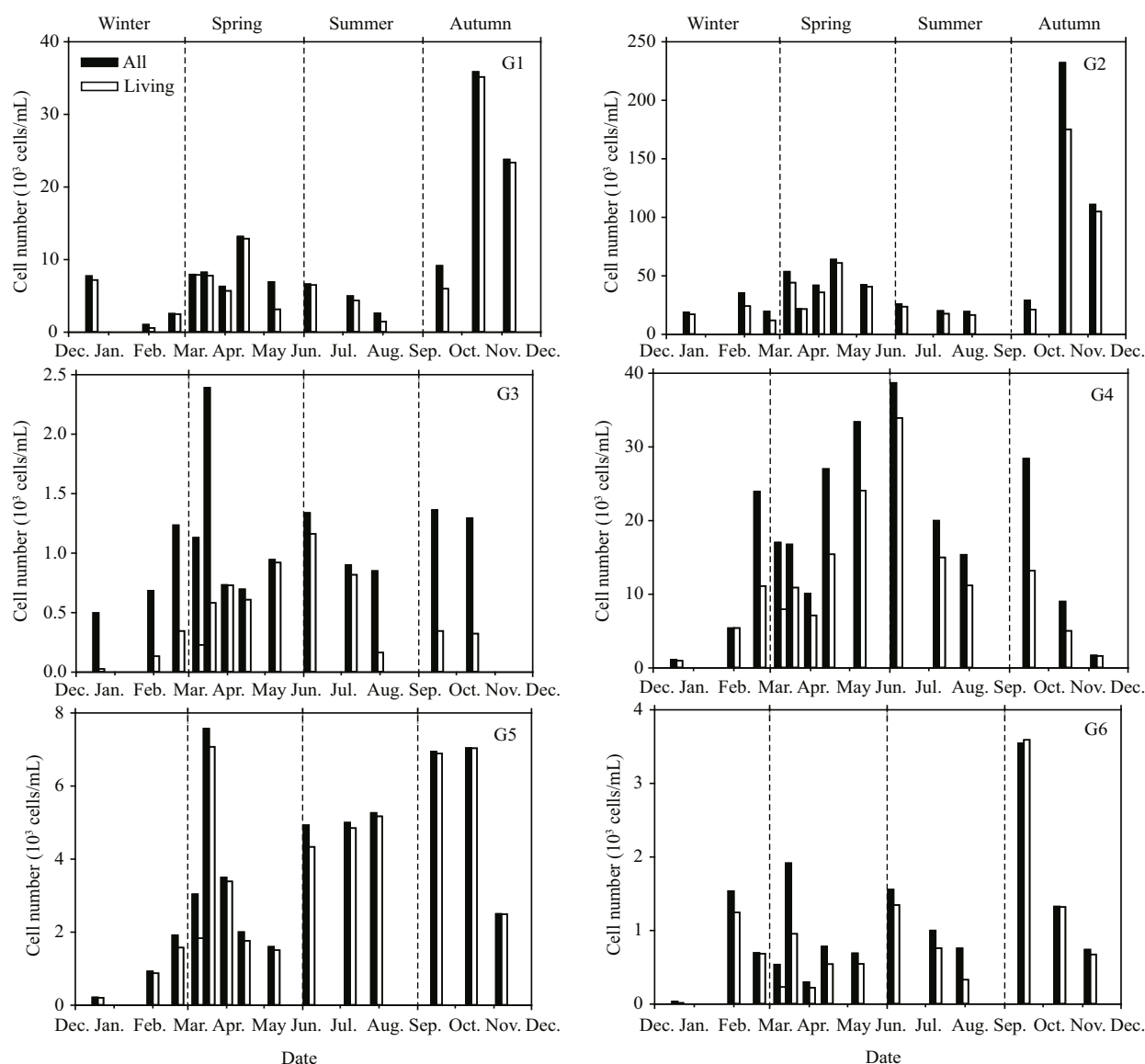


Fig.4 Variations in all cell (black bars) and living cell (open bars) abundance in each of the six flow cytometry derived phytoplankton groups from December 2012 to December 2013

abundance in October 2013 (Fig.4). G4 was the second most abundant with a general pattern of high abundance from spring to autumn and lower in winter, and accounted for 41% of total phytoplankton in summer (Figs.4 and 5). G4 AC abundances decreased with increasing salinity (Pearson $R=-0.666$, $P<0.05$; Fig.6). G5 AC abundances were positively correlated with temperature (Pearson $R=0.580$, $P<0.05$; Fig.6). While, G1 AC abundance was significantly, positively correlated with PO_4^- (Pearson $R=0.786$, $P<0.05$; Fig.6). Notably, Chl *a* concentrations were not significantly correlated with all cells of any group, but were significantly positively correlated with G4 and G6 living cells (Fig.7).

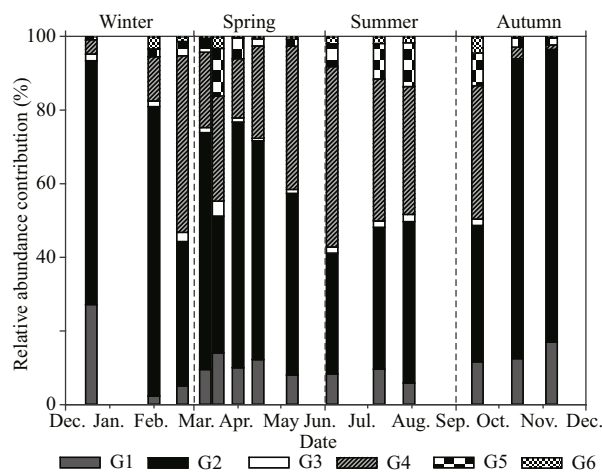


Fig.5 Contributions of the six groups (G1–G6) to total cell abundance from December 2012 to December 2013

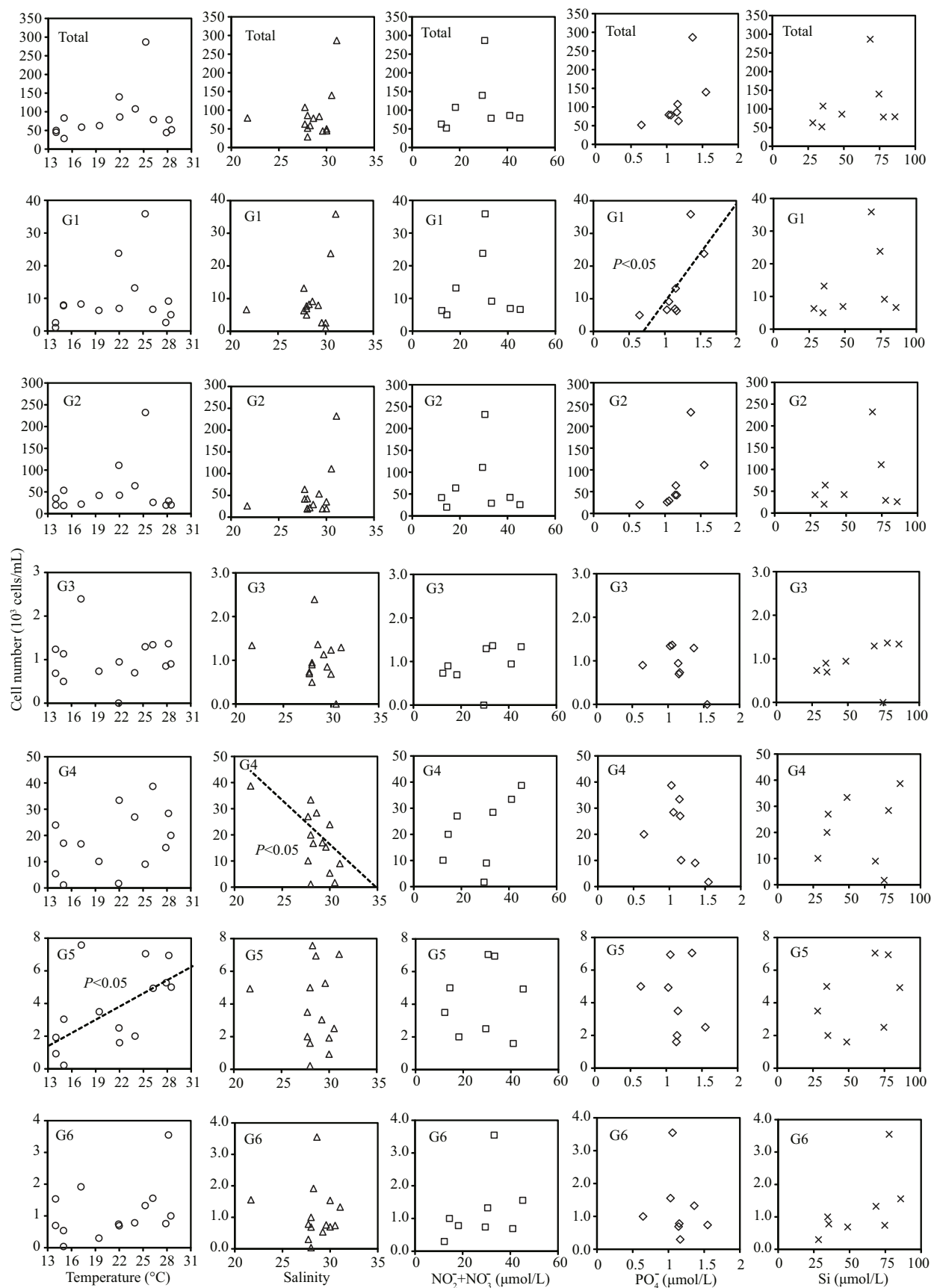


Fig.6 Relationship between the all cells of six groups (G1–G6) and environmental parameters

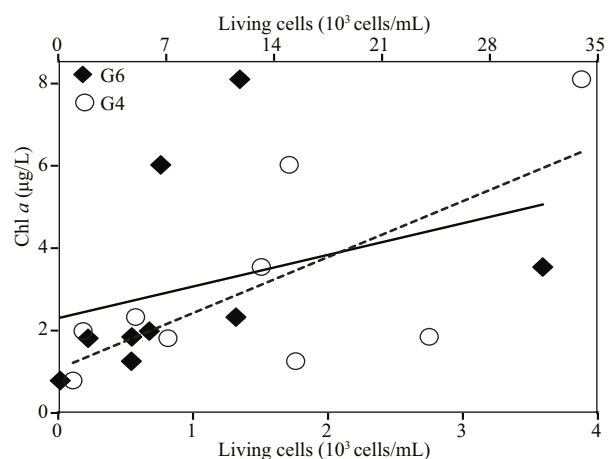


Fig.7 Correlation between Chl *a* concentration and living G4 (circles and dashed line) / G6 (black rhombus and thin line) cells

3.3 Percentage of dead cells

The percentages of dead cells (% DC) of different phytoplankton groups did not follow the abundance distribution and covered a wide range (Fig.8). In general, mean total phytoplankton % DC (5%–44%), which was high in winter (27%±16%) and low in summer (16%±6%; Fig.9), was inversely correlated with Chl *a* concentration (Fig.2f). There was no obvious regularity in G1 and G4 % DC, while G4 dead cells were the major component of total dead cells in spring and summer (54%; Figs.8 and 10). G2 % DC values were high in winter and autumn (maximum=40%), accounting for 61% and 70% of total dead cells, respectively. All G2 % DC values in spring and summer were below 20%, only accounting

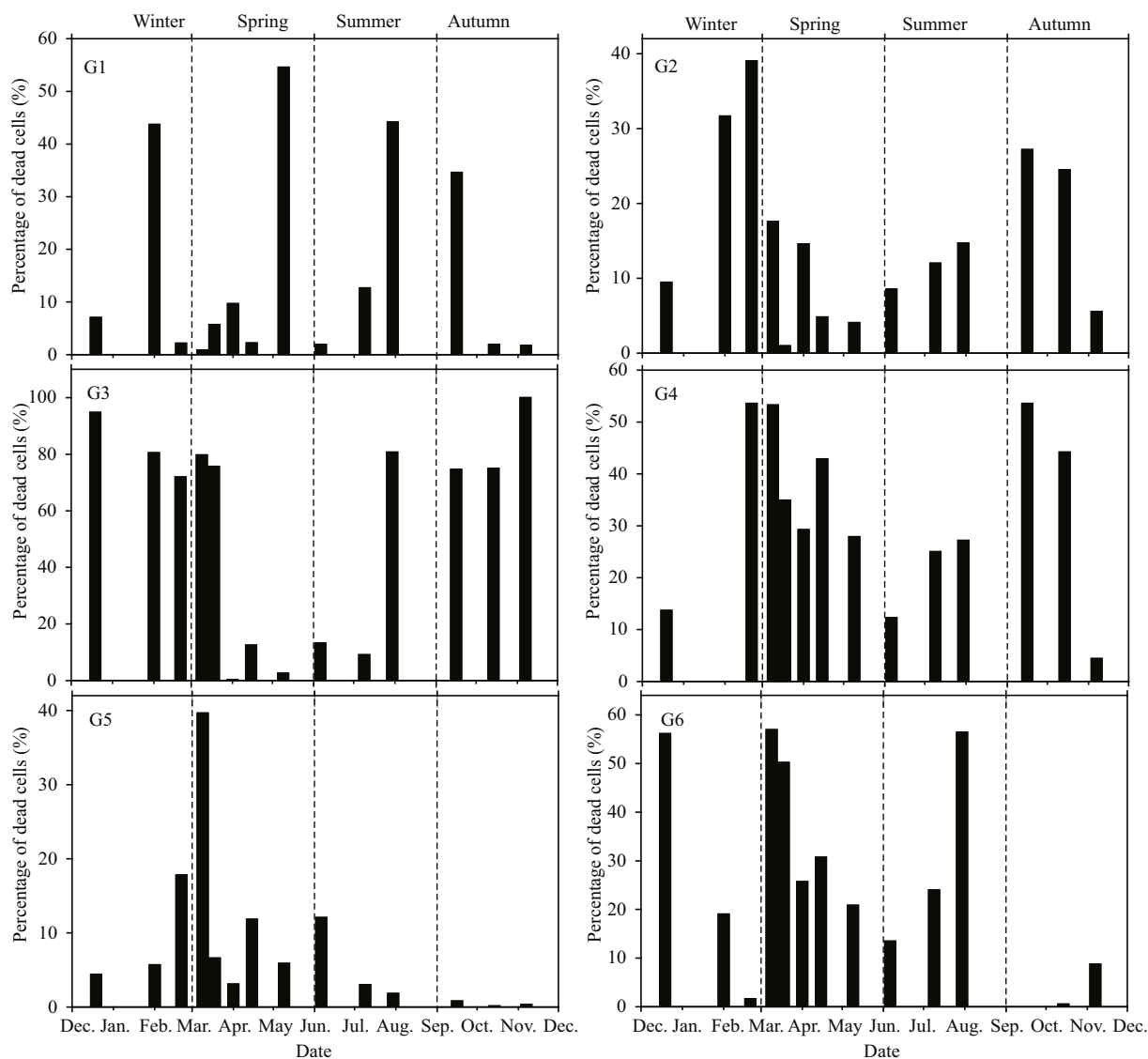


Fig.8 Variations in the percentage of dead cells (black bars) in the six phytoplankton groups from December 2012 to December 2013

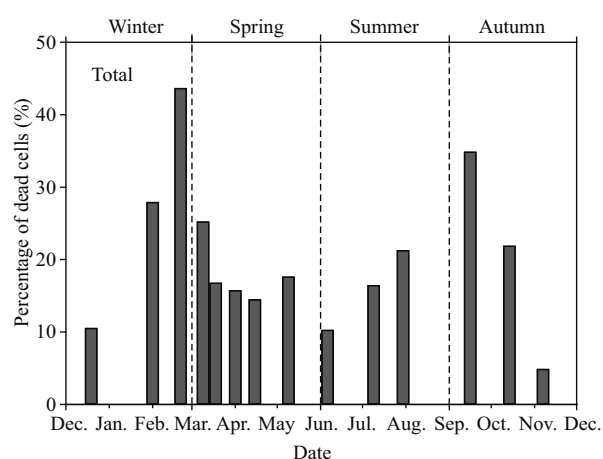


Fig.9 Variations in the percentage of dead cells in total phytoplankton from December 2012 to December 2013

for 28% total dead cells in both seasons (Figs.8 and 10). Except for April–July, G3 % DC values were significantly higher (70%–100%) than that of the other groups (Fig.8), and % DC was significantly negatively correlated with salinity (Pearson $R=0.598$, $P<0.05$; Fig.11). G5 % DC values were $<20\%$, except in March, and almost all G5 cells survived in the autumn (Fig.8). G6 % DC values decreased with increasing Si concentration (Pearson $R=-0.836$, $P<0.05$; Fig.11).

4 DISCUSSION

The importance of phytoplankton cell death is being increasingly recognized (Bidle and Falkowski, 2004; Franklin et al., 2006; Rychtecký et al., 2014). One characteristic of dead cells is loss of the ability to maintain homeostasis, resulting in increased cell membrane permeability (Darzynkiewicz et al., 1994). This characteristic is, therefore, used to identify dead cells. Techniques based on vital stains are used to identify and quantify living/dead phytoplankton cells (Onji et al., 2000; Peperzak and Brussaard, 2011). However, some cells either cannot be stained (e.g., *Synechococcus* sp.), do not stain incompletely (e.g., *Phaeodactylum tricornutum*). Furthermore, some stained cells are not bright enough or a strong background fluorescence is observed (Agustí and Sánchez, 2002; Peperzak and Brussaard, 2011). It is also difficult to identify phytoplankton cells from heterotrophic organisms after staining; stain fluorescence and autofluorescence can either interfere with each other or autofluorescence can overshadow stain fluorescence (Agustí and Sánchez, 2002; Garvey et al., 2007; Peperzak and Brussaard, 2011). Peperzak

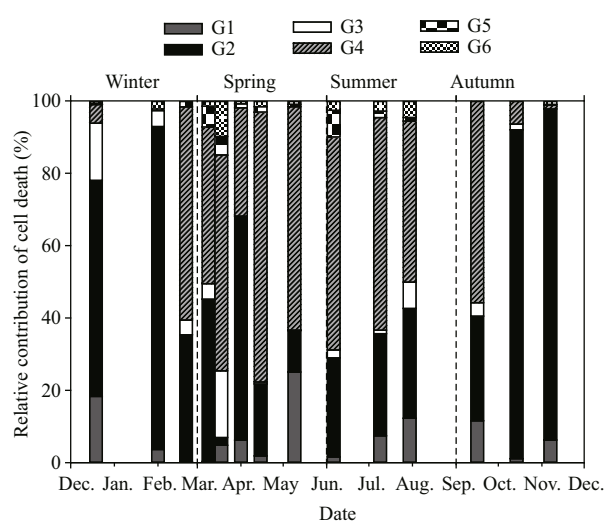


Fig.10 Contributions of the dead cells in the six groups (G1–G6) to total dead cells from December 2012 to December 2013

and Brussaard (2011) demonstrated that fluorescein diacetate (FDA) and acid-specific dyes (SYTOX-Green), which offer a wide range of vitality probes in various phytoplankton from different regions and at different stages of growth, cannot stain *Synechococcus* sp. Agustí and Sánchez (2002) reported that FDA cannot be applied to *Synechococcus* sp. and *Prochlorococcus marina*, whereas a cell digestion assay can. The cell digestion assay is a non-staining membrane permeability test to effectively quantify dead cells in culture or natural phytoplankton communities, and it has been applied to a variety of phytoplankton communities from different regions (Darzynkiewicz et al., 1994; Agustí and Sánchez, 2002; Agustí et al., 2006). This cell digestion assay only requires the incubation of samples for 45 min using Trypsin and DNase I to remove the dead cells from the population (Darzynkiewicz et al., 1994; Agustí and Sánchez, 2002). In the Xiamen waters, pico-phytoplankton is an important group that contributes approximately 10% Chl *a* in all seasons (Cao et al., 2005), and phycocyanin-rich cyanobacteria (PC) dominate (78% in Oct. 1997) in the western Xiamen waters (Huang et al., 2000). Therefore, we used a cell digestion assay to study phytoplankton cell death in Xiamen Bay.

Based on their size and fluorescence characteristics, G1 and G2 were considered cyanobacteria, and G2 was considered *Synechococcus*. Although, G2 cell numbers were the highest of all six groups, they contributed very little to the Chl *a* concentration. G2 % DC was high in winter (up to 29%) and low in

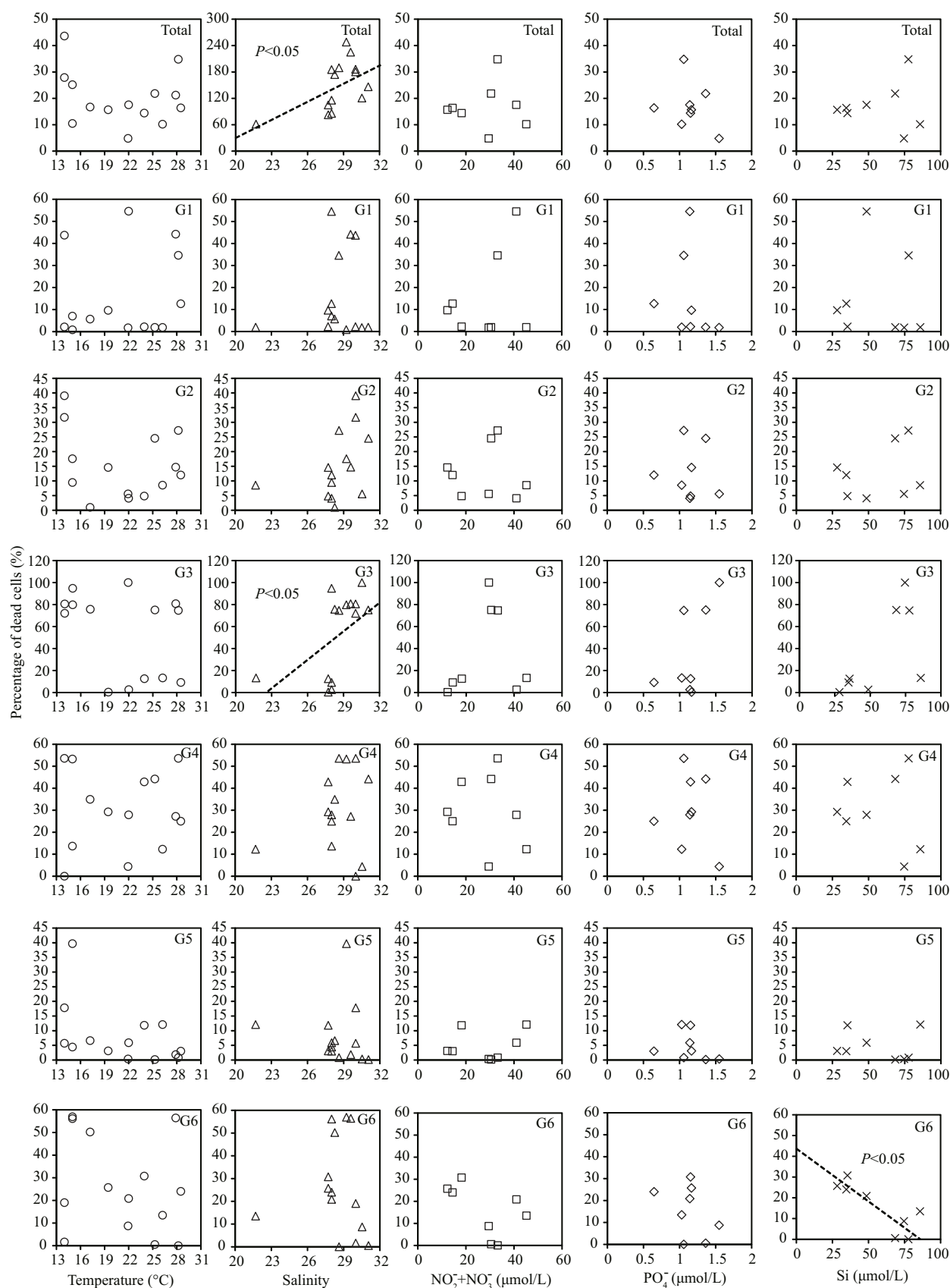


Fig.11 Relationships between the percentage of dead cells / total phytoplankton in the six groups (G1–G6) and environmental features

spring (only 8%), and the group contributed most to total dead cells in winter (61%), indicating that low temperatures could suppress *Synechococcus* growth and influence their viability in the ocean (Figs.8 and 10). This result was in agreement with those reported for the subtropical NE Atlantic Ocean Water where temperature was an important factor influencing the growth and distribution of pico-cyanobacteria (Alonso-Laita and Agustí, 2006). G3 cells appear to be sensitive to both temperature and salinity, and they die easily with increasing salinity and decreasing temperature (Figs.2a, b, and 8). G3 % DC was significantly higher than that of the other groups in winter and autumn, ranging 70%–100% (Fig.8). This suggested that cell death, as a major source of loss, is an important process regulating the seasonal variability in G3 abundance in this area. G6 had the biggest cells, and its % DC was negatively correlated with silicon concentration (Fig.11). Hence, the majority of G6 cells were large diatoms, which was verified by microscopic analysis. A proportion of G4–G6 % DC was >50%, and this further indicated that cell death can regulate phytoplankton community abundance.

Considerable variability in dead cells was observed among phytoplankton groups and cell death varied seasonally (Fig.8), which was also reported by Agustí and Sánchez (2002). The variation tendency of mean total phytoplankton % DC, which was high in winter and autumn and low in spring and summer, was inversely correlated with Chl *a* concentration (Figs.2f and 9). This finding was similar to that of a previous study in seven Florida lakes where Chl *a* concentrations were significantly positively correlated with average percentage living cells for all groups (Agustí et al., 2006). Moreover, our study also showed that Chl *a* concentration was significantly, positively correlated with living cells in groups G4 and G6, but not with any other group. Therefore, explication of activity parameters (such as Chl *a*) are more meaningful if they can be related to viable cells rather than total cells (Garvey et al., 2007).

Our sample site is in a subtropical area with high water temperatures, full solar radiation, and is nutrient rich. Therefore, Chl *a* is high all year round. In general, nutrients were replete for phytoplankton growth and were not major factors determining phytoplankton distribution at this station. The main factors affecting phytoplankton distribution were temperature and salinity. Our results showed that only silicon was a key determinant for the viability of G6

(mostly large diatoms). Other nutrient concentrations had little influence on phytoplankton death (Fig.11); as with phytoplankton distribution, the main factors affecting phytoplankton death were temperature and salinity. The different % DC patterns for the various groups suggested that phytoplankton differ greatly in their capacity to cope with various stressors (e.g., silicon concentration, low temperatures, and high salinity). Indeed, some laboratory and field studies have indicated that phytoplankton cell death decreases from oligotrophic oceanic stations to more eutrophic waters, and the various species have different physiological responses to environmental constraints (Brussaard et al., 1997, 2005; Lee and Rhee, 1997; Brussaard and Riegman, 1998).

The lowest average % DC value at our site was $16\% \pm 6\%$ in summer, despite it being in a subtropical area. In the eutrophic coastal waters of the subtropical NE Atlantic, the percentage of nano- and micro-phytoplankton dead cells reached 20% (Alonso-Laita and Agustí, 2006). In the oligotrophic open ocean of the subtropical NE Atlantic, the percentage of nano- and micro-phytoplankton dead cells reached 81.4%–88.2% (Alonso-Laita and Agustí, 2006). Hence, phytoplankton cell death is a process that cannot be ignored when studying aquatic ecosystems and biogeochemical processes. Most population biologists, who study predator-prey dynamics using common research tools, such as nutrient-phytoplankton-zooplankton (NPZ) models (Fussmann et al., 2000), do not consider prey (phytoplankton) mortality. Moreover, ignoring prey cell death may explain why there are discrepancies between their models and the experimental results. Therefore, cell death has broad consequences for the functioning of aquatic ecosystems. Studying this process can help to understand phytoplankton population dynamics and the evolution of community structure.

5 CONCLUSION

In summary, the cell digestion method applied here successfully identified and quantified dead/living cells at our sampling site in Xiamen Bay. Our results provide evidence for large seasonal variations in dead cells among various phytoplankton taxa, and various species have different responses to environmental constraints. Temperature and salinity are the key determinants of phytoplankton distribution and mortality in Xiamen Bay. Phytoplankton cell death is a key parameter affecting phytoplankton population dynamics, and identification and quantification of

dead/living cells is more meaningful than using total cells to explicate Chl *a*. Therefore, cell death has broad consequences for subtropical ecosystems and biogeochemical cycles. Thus, cell death cannot be ignored in future studies.

6 ACKNOWLEDGMENT

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